Home Office

Animals (Scientific Procedures) Act 1986

Non-technical summaries for project licences granted July - December 2022

Contents

1. The mechanisms underpinning ‘steroid’ (glucocorticoid) development of obesity and diabetes

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Steroids, Glucocorticoids, Diabetes, Obesity, Brain

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Synthetic glucocorticoids, commonly called steroids, are used as medicines to treat a wide range of illnesses such as asthma, rheumatoid arthritis, and multiple sclerosis. Although well tolerated, long term, high dose use can lead to side effects including obesity and diabetes. Therefore, the aim of this study is to identify how longer term use of glucocorticoids acts in specific parts of the brain to increase food intake, and to alter processes in the body leading to high glucose and diabetes. Our long term aim is to find alternative synthetic glucocorticoids or alternative ways of administering them to reduce the associated obesity and diabetes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Glucocorticoids (steroids) are a widely used class of medicines. They are anti-inflammatory and used to treat diseases such as asthma, arthritis and autoimmune conditions such as multiple sclerosis as well as in the treatment of cancer. With long term, high dose use, patients can develop side effects similar to those seen in the metabolic syndrome, a cluster of illnesses associated with obesity and diabetes. These include weight gain, especially around the waist, type 2 diabetes, and fatty liver disease. These side effects can limit the use of an otherwise good and inexpensive medication. In this body of work, we want to understand how the glucocorticoids cause the side effects. By understanding this, it might be possible to find other glucocorticoids that don't cause the side-effects or medicines to be given with the glucocorticoids that decrease the side effects. This would mean that more patients could be treated with these medicines.

What outputs do you think you will see at the end of this project?

At the end of the project we will have gained new information about how glucocorticoid treatment causes side effects such as obesity and diabetes. Diabetes is a serious, longterm illness, that often requires daily medication. Furthermore, when not well controlled, diabetes can increase the risk of kidney disease, blindness and cardiovascular disease, including heart attacks and strokes. Obesity is a condition, which itself can lead to diabetes, but can also increase the risk of certain cancers. All of these are life limiting for the patient and the complexity of the treatment decreases their quality of life and makes it very expensive for the NHS.

The information generated from this proposal will be shared in research papers and with other scientists and doctors at conferences. If we can understand how the side effects develop then we, with doctors and the pharmaceutical industry, will be able to design new types of glucocorticoids which don't cause the problems or different treatments given with the glucocorticoids to reduce the side effects.

Who or what will benefit from these outputs, and how?

The main beneficiary of this work will be patients treated with glucocorticoids and their doctors who are able to prescribe them with reduced worries about side effects. The NHS will also benefit, as glucocorticoids are cheap medicines and will be able to be used more widely. Additionally, the costs of treating the side effects such as diabetes are also high, so these may be reduced if the side effects of glucocorticoids are reduced. The scientific community will also benefit from the outputs generated in this project, as we will be increasing the knowledge of how these steroids work.

How will you look to maximise the outputs of this work?

Previously, we have collaborated with groups at other universities, with pharmaceutical companies, and with clinical endocrinologists who see patients with these side effects. Therefore, we are well placed to contact those groups of people who can use the outputs of our studies most effectively. These collaborations have not only been for exchange of knowledge, but also to learn the most refined way of carrying out the experiments. We will present our early data at conferences, so fellow scientists and doctors can comment on the experiments while they are progressing. This will ensure that the correct studies are being carried out. We will also publish our data, whether successful or not, in open access

scientific journals as well as sharing our new publications on social media platforms to increase the visibility. All raw data will be made available upon request.

Species and numbers of animals expected to be used

- Mice: 10500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

For our research we use an adult mouse model, which is treated with glucocorticoids (steroids). This model is able to mimic the side effects of the treatment that are seen in humans. Also, the pathways in the development of these side effects of obesity, diabetes and fatty liver disease are thought to be similar between humans and mice. In addition, we are looking at actions in the brain so we need to collect brain tissue and we look in mice bred with certain pathways removed, making these animals the best model for our investigations.

Typically, what will be done to an animal used in your project?

Most mice in this project will be treated with glucocorticoids to look at the side effects of diabetes and obesity. Typically, we give this treatment in their drinking water to reduce the stress of handling and the short term pain of an injection. They will be weighed frequently and depending on what we are looking at they may have a glucose tolerance test, where they will be fasted before they are injected with glucose and small blood samples taken from the tail. Alternatively, they may have their metabolic rate measured by placing them in special cages that can measure this. We also may look at how much fat they have using a type of MRI scan, but as it doesn’t give an image, they don’t need to stay as still. Before the end of the study, we take blood samples from the tail to measure things such as stress hormones and insulin. Many of our protocols look at changes in factors associated with appetite and feeding in the brain, but these measurements are made after the study finishes.

Some studies will be carried out where we need to give drugs directly into the brain. For these studies, mice will undergo a short surgery with a quick recovery anaesthetic and a device will be inserted, so these drugs can be directly administered to the brain when the mice are awake and freely running around. During and after surgery, mice will be given appropriate pain relief, kept warm and given soft food to help them recover as quickly as possible.

What are the expected impacts and/or adverse effects for the animals during your project?

With glucocorticoid treatment the mice should gain weight and become overweight or obese. The treatment period for these studies are 4 weeks at their longest, so the animals are just at the start of developing diabetes. Some will develop diabetes and therefore drink more and urinate more, but we will change their bedding more frequently to account for

this. As the measurement of food intake is important for our research, animals will often be in a cage on their own, which can disrupt their normal behaviour, but we provide them with tubes to play and hide in and additional bedding and chew sticks to reduce the impact of this. The total length of time where the animals would be housed alone is generally 7 weeks (1 week to acclimatise, 2 weeks to take normal measurement and then 4 weeks on treatment). A very few studies may want to investigate genetic changes related to glucocorticoids in mice and then mice may need to be housed alone for up to 6 months.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most animals will have a mild experience. They will eat more and will gain weight, but not to the point that it impacts their welfare. There could be transient pain from injections or blood sampling. There may be a mild stress effect of social isolation.

The animal having surgery will have a moderate severity experience. They will be given quick recovery anaesthetics to reduce the impact of the anaesthetic, painkillers to reduce any pain and will be kept warm until fully recovered. Mice tend to recover quickly from this surgery.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We look at the effects of glucocorticoids (steroids) on the whole of the body. We mainly give these to the animals in their drinking water, which is similar to patients swallowing tablets, and we look at their effects on multiple organs in the body, such as fat, liver and muscle. It is not possible to mimic these effects in dishes of cells as you cannot look at the overall effect on the body.

Additionally, we look at how glucocorticoids acting in the brain then signal to the other organs in the body. As we are looking at how one organ in the body controls others, a whole body system is needed for this.

We have found from our previous studies that mice respond similarly to patients when given these steroids, so they are used in our studies.

Which non-animal alternatives did you consider for use in this project?

A potential alternative is to work on neurons growing in culture. However, these neurons do not survive and do not develop into cell lines. There is a technique for adapting them to

grow continuously which is complex but possible, and this offers a way of addressing specific questions about the neurons that regulate food intake. Several years ago, we obtained immortalised hypothalamic neuronal cell lines from a collaborator to determine if they produced a key neuropeptide regulating food intake. Despite a lot of effort in culturing the cells, we were unable to get these neuronal cell lines to synthesise this neuropeptide. We subsequently worked with another collaborator who uses embryonic stem (ES) cells differentiated into neurons and studied the production of these neuropeptides. As the neuropeptides are a glucocorticoid (steroid) target in the brain, we will be able to assess the effects of chronic glucocorticoid treatment on these peptides in the ES cells. This would complement the *in vivo* studies where we consider the effects of glucocorticoids on food intake.

Why were they not suitable?

As stated above, neurons which were adapted to grow continuously did not produce the neuropeptides, but we have subsequently collaborated with a research group using ES cells which could provide key information on the effects of chronic glucocorticoid treatment. However, there is growing evidence that these neurons are quite heterogeneous in the brain and they act at several different target neuronal populations which would be impossible to mimic *in vitro*.

In addition, to prove that glucocorticoids are acting via these specific neuropeptides to modify food intake, body weight and peripheral metabolism, we will still need to do these studies *in vivo*. We also need to study the effects of glucocorticoids on multiple body tissues such as liver, adipose tissue and muscle at the same time, to examine the molecular changes underpinning the side effects in patients treated with these medicines. Therefore, single cell types will not inform us of the overall effects on the body.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Generally, we use the data from previous studies to help us estimate the number of animals required for an experiment. This allows us to use the minimum number of animals to give us a reliable answer to the question. Where it is a completely new experiment, we will use a small group size at the start, so we can monitor how many animals we will need.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To design the experiments we use the NC3Rs experimental design assistant to get the best study design and to use the correct number of animals. At the end of the study we take organs from the animals that we might not necessarily need at the moment, but so we wouldn't need to run another study in the future if we need to look at the effects of treatment that organ.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We breed the minimum number of animals possible for our projects that will give us the best experimental results. When we start new studies, we carry out pilot studies to understand how the animals will react and to help us estimate the number of animals we will need. At the end of the study, we take as many tissues and organs as possible, to minimise the need to run later studies, we also share the tissues from some studies with other groups, so they don't need to run their own experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This licence application only uses mice, the least sentient animal possible for our studies. Mice are used as they give similar responses to glucocorticoids (steroids) as is seen in patients treated with these medicines. We mostly give the glucocorticoids in the drinking water, so the animals don't need to be handled and stressed as much and this approach reduces the need for multiple injections. None of the mice with genetic changes show any side effects to these gene changes.

Why can't you use animals that are less sentient?

We have investigated zebrafish models and there are some good examples of novel data describing how glucocorticoids act within the cells. There has also been information on how glucocorticoids control development of the zebrafish. However, the systems are too limited to be used to study whether the glucocorticoids are acting in the brain or directly in organs in the body that regulate metabolism. In addition, there seem to be key differences in zebrafish in the expression of enzymes that activate/inactivate glucocorticoids. We have also investigated what is known in zebrafish about the melanocortin system, which is a target of glucocorticoids that this project addresses. Unfortunately, previous work has only studied a rudimentary peptide system in another organ and there is no obvious melanocortin system in the brain to work on.

Therefore, mice are the least sentient animals that mimic the glucocorticoid induced changes that lead to obesity and diabetes as seen in human patients. As the glucocorticoid effects which lead to obesity and diabetes develop over time, we cannot do these studies in terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The animals in our studies are already closely monitored to ensure they suffer the minimum pain and harm. The small number of animals that undergo surgery get pain medication, and are fed soft food and kept warm after the operation to minimise any pain and suffering. Where animals need injections, the mice will be handled to get them used to this prior to the experiments. We also keep ourselves updated so we are informed of any improvements that we can implement to minimise any harm to the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the PREPARE guidelines as well as any advice we receive from the statisticians, vets and NACWOs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To keep informed of advances in the 3Rs, we regularly check the NC3Rs website. We also receive updates from our animal unit and our NTCO about any changes. We also attend the workshops organised by the NC3Rs and our university to keep informed of new advances in animal welfare and the 3Rs.

2. Novel Protein Targets for Cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, therapy, brain, metastasis

Animal types	Life stages
Mice	adult, neonate, juvenile, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to establish novel proteins as prognostic markers and possible therapeutic targets in primary brain cancer and brain metastasis. The proteins have many targets in brain cancer cells and state-of-the-art techniques will be used to elucidate the mechanism of how the proteins trigger brain cancer and/or metastasis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Brain cancer is a major unmet need with 5 year survival being 10% only. Identifying a new target in brain tumours might pave the way for further understanding of how the tumours progresses/metastasizes and may be arrested therapeutically.

What outputs do you think you will see at the end of this project?

Establishment of a novel drug target in cancer. We expect multiple publications, datasets and future drug discovery endeavours arising from this study.

Who or what will benefit from these outputs, and how?

Glioblastoma multiforme (GBM) is a highly invasive brain cancer with very poor patient 5-year survival. Since identifying therapeutic interventions for GBM is a major unmet need, multiple scientific groups around the world are focused on this disease. A simple keyword search on pubmed.gov reveals that there are over 39,000 publications on GBM, with over 230 in 2020 itself, while clinicaltrials.gov reports over 480 active GBM clinical trials. Furthermore, finding a kinase (a type of enzyme) that is involved in promoting GBM and could be targeted by drugs would have considerable interest to basic scientists working on how the immune system is controlled, the effects of genes, how messages are sent between and within cells and how cancer occurs It will also be of interest to translational scientists working on the mechanisms of carcinogenesis, pharmacology and biomarkers, through to pathologists and medical oncologists involved in managing patients with brain cancer. Those engaged in medicinal chemistry and personalized medicine may find the results helpful to their work and the ultimate aim is to help patients.

The findings of the project will clearly benefit basic scientists, clinicians, oncologists, and patients.

The project will generate novel data about the prognostic and diagnostic significance of biomarkers in GBM.

The project will also identify mechanisms of how cancer spreads to the brain from the breast.

How will you look to maximise the outputs of this work?

My collaborators in USA will also be involved in developing tools for research and will be in a position to disseminate tools and knowledge to the scientific community in USA and beyond. The fact that the proposal requires a diverse range of state-of-the-art techniques available at our establishment, makes it probable that most of the academic areas that would benefit from the research will be covered when our findings are disseminated at scientific conferences and at seminar presentations. Moreover, the research findings will be published in open access journals to allow anyone to see it.

Species and numbers of animals expected to be used

- Mice: 6100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The initiation and progression of cancer involve a complex interplay between genetic mutations, the cell-of-origin and its microenvironment, cytokines (inflammatory molecules) and growth factors, and the extracellular matrix (the environment the cells are suspended in). In some cases, development of the disease is a multi-step process that occur over months. It is therefore a very difficult process to study in a cell culture system, because we can't re-create all the environmental conditions that might influence how cancer develops and so may not get accurate results. Even the most sophisticated cell culture systems existing today will select for specific properties in a heterogeneous (mixed) cell population and allow certain cells to grow, while other cells, which might be more relevant to the disease don't or grow poorly. This is particularly true in the study of brain cancer where the extracellular matrix is composed of vastly different components as compared to any cell culture. It is therefore essential to use the brain of a whole organism to properly and faithfully reproduce the disease development and maintenance and make discoveries that can help patients suffering from cancer. Onset, maintenance and progression of cancer are complex biological processes that cannot be replicated in lower organisms. Pre-cancerous and fully malignant cells are highly dependent on interactions with the microenvironment and the extracellular matrix and must be considered in the context of the whole organism. The mouse brain is evolutionarily closely related to human, mice are relatively easy to breed, and the mouse genome is amenable to genetic modification to introduce mutations that occur in man. Therefore, adult mice represent the best available model organism for studying cancers.

Typically, what will be done to an animal used in your project?

Animals with specific alterations in the genes which encode the proteins of interest will be bred. Tumour cells will be implanted into these mice and "wild-type" animals (mice which do not bear these genetic alterations), either under the skin or by injection through a small hole in the skull into a specific region of the brain. When we are studying tumour metastasis (spread), the original tumour under the skin may be removed surgically, while we look for deposits appearing in the brain.

What are the expected impacts and/or adverse effects for the animals during your project?

The surgical procedures to create the hole in the skull for injections and to remove any primary tumours could cause pain, but this will be prevented by giving analgesics before and after the surgery, as advised by the vet, and general anaesthesia during the procedure. Tumour development generally leads to weight loss and loss of appetite. Animals will be euthanased before any further welfare issues can arise.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

As we are studying tumour development, most of the animals being studied, unless they are control animals, will develop tumours. Analgesia is given for surgical procedures. Any mouse exhibiting early stages of discomfort from the tumour will be killed and organs harvested for further analysis. Where we give drugs, these will have been shown to be safe and so should have no side effects. Overall, as we will use early humane endpoints,

we expect 20% of mice to exhibit moderate severity, with the remaining animals exhibiting mild severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The development of glioblastoma multiforme (GBM) is a complex multi-step process that occurs over months. Onset, maintenance and progression of GBM are complex biological processes that cannot be replicated in lower organisms or cell cultures. Pre-cancerous and fully malignant cells are highly dependent on interactions with the microenvironment and the extracellular matrix and must be considered in the context of the whole organism. It is therefore essential to use the brain of a whole organism to faithfully recapitulate the disease development and maintenance and make discoveries that can help patients suffering from GBM. The mouse brain is evolutionary closely related to human, mice are relatively easy to breed, and the mouse genome is amenable to genetic modification to introduce mutations that occur in man. Therefore, mice represent the best available model organism for studying brain cancers, especially GBM.

Which non-animal alternatives did you consider for use in this project?

Brain cancer cell lines

Why were they not suitable?

Initial proof-of-concept studies will be carried out in patient-derived GBM cell lines. However, onset, maintenance and progression of GBM are complex biological processes that cannot be replicated in lower organisms or cell cultures.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We estimate using 600 animals per year in maintaining the breeding colony and another 620 animals, some of which will have come from that colony, in experimental procedures; a total of 1220 animals per year, or 6100 over the expected term of the licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During experimental design, we utilised power calculations and also previous literature in the field to ascertain sample size. We also utilised the NC3R's experimental design assistant to ascertain sample size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Power calculations will be carried out to ascertain sample size in collaboration with a statistician. Standard immunocompromised genetically altered mouse lines will be purchased from commercial suppliers, but the lines with modifications in the genes of specific interest are not commercially availabe and will therefore be bred in-house. I am experienced in colony management and will use strategies, e.g. those outlined in the NC3Rs Efficient Breeding of Genetically Altered Animals pages, that help to minimise numbers bred. I will take advice on breeding strategies from the NVS (Named Veterinary Surgeon) and NACWO (Named Animal Care and Welfare Officer) responsible for the breeding unit, as required. I will share lines of interest with collaborators and will “batch” breed to allow shared controls for studies where appropriate.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

There are three main models which we plan to use. All these models allow us to monitor the tumour growth in mice with minimum harm as appropriate early humane endpoints can be identified.

1. Ectopic tumour growth wherein cancer cells are injected under the skin in an area where the tumour won’t cause any pressure on the surrounding tissue but will allow tumour growth and tumour measurements using callipers to be easily monitored. We will ensure that animals are killed before the tumour causes any outward signs of discomfort.

2. Cancer cells are injected as in (1), with the intention that some will migrate to the brain (a model of metastasis). The original tumour may be removed surgically, so as not to grow to a size that might cause welfare problems. Mice are expected to recover completely in a very short space of time after the surgery. Tumour growth in the brain may lead to weight loss and loss of appetite, but we will monitor animals carefully for these (or any other signs of illness) and animals can be killed humanely as soon as they become apparent.

3. Intracranial injection is where we will implant tumour cells directly into the brain of anaesthetised mice, as a model of primary brain cancer. Mice are expected to recover completely in a very short space of time after the surgery. Tumour growth leads to weight loss and loss of appetite, but we will monitor animals carefully for these (or any other signs of illness) and animals can be killed humanely as soon as they become apparent.

Why can’t you use animals that are less sentient?

We need to monitor tumour growth in live animals which are closely related to human physiology. Mice and human brain are quite alike when it comes to supporting tumour growth while immunocompromised and well established tumour mouse models exist which allows easy execution and interpretation of scientific work. Less sentient or immature animals would not be ideal since they will have neither the matured organs to support the tumour nor will they exhibit the complex blood- brain barrier phenotypes typical of adult mammals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Analgesic will be administered prior to any surgery, as advised by the vet. Mice will be under anaesthesia for all surgical procedures (including the intracranial administration and the injection of tumour cells into the mammary-fat-pad). All surgery will be performed under strict aseptic conditions and mice will be monitored carefully for any evidence of post-operative pain or discomfort, as well as for signs of tumour development. Drugs given will be non-toxic and the route of dosing, volumes and injections will be those expected to cause the least discomfort possible for the study aim.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The Nc3R guidelines and power calculations from pilot studies will be used to refine all experiments. Published works on the models will also be utilised.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Regular online reading on the 3R websites (such as the NC3Rs), remaining up to date with the literature in the field of research and departmental emails and alerts, including advice from the Named Information Officer (NIO) and Named Veterinary Surgeon (NVS)/ NACWO (Named Animal Care and Welfare Officer).

3. Vascular-Mesenchymal Crosstalk in Bone

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

bone, regeneration, chemotherapy, radiation, blood vessels

Animal types	Life stages
Mice	adult, juvenile, neonate, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to explore new ways to promote bone regeneration

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The proposed research will aim to identify novel targets to promote bone regeneration. Musculoskeletal disorders affect approximately 50 % of the human population, thus posing a significant health, psychological and economic burden on healthcare systems. Further regeneration of the bone and bone marrow microenvironment after cancer therapy still remains a problem. Identification of cellular and molecular drivers of bone regeneration would be of immense value in the clinic to prevent, manage and treat bone-loss and bone diseases.

What outputs do you think you will see at the end of this project?

Although there have been advancements in our understanding of vascular and skeletal dynamics in the bone microenvironment, a comprehensive understanding remains elusive. Primarily, our research will explore the cellular and molecular players, involved in bone regeneration. This will further add to existing biological knowledge, scientific publications, and identification of novel therapeutic targets to improve disease outcome and thus patient prognosis.

Who or what will benefit from these outputs, and how?

The work detailed in this project will facilitate healthcare industries to develop improved therapies to prevent, manage and treat bone diseases. Therefore, the primary beneficiaries will be the patients suffering from bone loss conditions. In addition, this research will result in the development of a new experimental system (bone vasculature) to an already multidisciplinary unit at the establishment. Being one of the pioneers in the field, my expertise will be beneficial to multiple scientific communities such as musculoskeletal, vascular, and regenerative biology. My research interests and expertise in bone vasculature will provide ample opportunities for scientific collaborations within the establishment and across the UK.

How will you look to maximise the outputs of this work?

1. Peer-reviewed, high impact and cutting-edge publications.

2. Presentation of the work in international conferences by my team members and me.

3. Publishing of negative data and unsuccessful experimental approaches.

4. Public engagement.

5. Dissemination of new findings through media and social media.

Species and numbers of animals expected to be used

- Mice: 11000 for 5 years.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

One of the key aspects of this study is to analyse the crosstalk between blood vessels and mesenchymal cells. The work described in this PPL requires the use of genetically modified adult mice to study the role of vascular niches in healthy skeletal bone versus during bone damage, i.e. following chemotherapy treatment. In the laboratory, we have availability to a wide variety of reagents suitable for mice to study clinical conditions.

Typically, what will be done to an animal used in your project?

To address the outlined research aims, our work requires the use of genetically altered mice which will bred under this project. Mice may receive substances such as Tamoxifen to alter their genetic makeup. Mice may receive a lethal dose of irradiation followed by reconstitution of the bone marrow via an injection of cells. Mice may then receive therapeutic agents to (drugs and inhibitors). Blood samples may be collected via cardiac puncture for further analysis. Mice may be injected with various therapeutics, e.g., chemotherapeutic agents, to investigate bone regeneration responses. Mice will then either humanely killed or undergo imaging under a terminal anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice that undergo irradiation are more susceptible to infection in the first 28 days following the procedure. This may manifest as weight loss, diarrhoea, or general malaise such as hunched posture or ruffled fur. Mice will be given antibiotics in their drinking water to reduce the risk of infection. Once past this time point no further adverse effects are expected. Mice that are given gene altering drugs may experience transient weight loss during the administration phase but are expected to recover quickly once this has been completed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- 50% Subthreshold
- 30% Mild
- 20% Moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal models are necessary to study the complex microenvironment in the bone marrow as currently other approaches, such as culture techniques, do not provide a realistic alternative. Furthermore, genetically modified mice provide the unique ability to study the role of specific genes in the complex environment of the living organism. We will aim to minimise the use of animal models where possible, whereby we will use the most refined animal model to address our research questions.

Which non-animal alternatives did you consider for use in this project?

Initial studies will be performed on cell-based assays using primary endothelial cells and differentiated cell populations. As, 2D studies are poor representative of in-vivo conditions, our aim is to interpret the physiological relevance of in-vivo cell behaviour by using 3D co-culture models.

Why were they not suitable?

Although in-vitro techniques such as cell culture can provide insight into specific cell types, these techniques cannot accurately replicate the physiological relevance and multifaceted environment in the bone tissue.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Mice numbers are calculated based on our previous data and experience. When required, we shall consult with statisticians within the department and apply statistical methods to determine the appropriate number of mice for each experiment. Alternatively, where experimental approaches are used routinely, previous experience will be used to determine the animal numbers used for the experiment, ensuring the least number of animals are used as possible.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Each experiment will be planned to adhere to the NC3R's experimental design. Experimental Design Assistant, a tool used to aid in efficient experimental design prior to embarking on the experimental work. Where possible other approaches, including cell-based culture systems, will be employed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

At every stage in our experiments, consideration will be given to ways in which we can reduce the number of animals. We will adhere to the following steps:

1. Appropriate and efficient breeding strategies.

2. Use stored samples, e.g., tissues acquired from other laboratories.

3. Store tissues from our experiments and provide them to other laboratories as required.

3. Perform pilot studies with a limited number of mice prior to embarking on larger experiments.

4. Where possible, use non-animal models such as 3D co-culture assays

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The physiological similarities between mice and humans make them an excellent model system for gaining insights into the regulation of the bone microenvironment in humans.

Moreover, they share similar endocrine systems to humans that allow us to extrapolate our findings to human disorders, including bone diseases. Irradiation may be used in to investigate role of blood vessels during bone remodelling and to study the regenerative potential of blood vessels cells. Lethal irradiation in all our experiments will be followed by an intravenous injection of cells to reconstitute the immune system. Irradiated mice engrafted with hematopoietic stem cells will be continuously monitored for signs of infection, anaemia or other symptoms of irradiation-induced illness (e.g., weight loss) till the end of the experiment. To visualise angiogenesis and follow the interactions of blood vessels with surrounding cell types and microenvironment by in-vivo microscopy will be performed under terminal anaesthesia. This in-vivo imaging approach will be crucial to determine the dynamics of blood vessel growth and the microenvironment. After induction of anaesthesia with volatile anaesthetics, surgery will be performed under general terminal anaesthesia. Mice will be anaesthetised to surgical depth/stage. Aseptic skin preparation will be performed; followed by an incision will be made on one of the bones (e.g., tibia, femur) to expose the bone surface for imaging.

Mice will be continuously monitored. All this will be performed under aseptic conditions. The mouse will be placed on a Biotherm stage warmer at 370C and imaged using a multiphoton microscope. The fluid will be administered and volatile anaesthetics induce and maintain the mice under deep anaesthesia. Either before or during intravital microscopy, mice may be injected with contrast dyes, fluorochromes or other imaging agents via an appropriate route to visualise blood vessels and surrounding cells. Transgenic mice expressing reporters such as GFP may be used to track blood vessels and pericytes. LASA guidance will be followed for aseptic surgery. In-vivo imaging will be critical in providing insights into the endothelial mesenchymal interactions in bone. Most importantly it will be done under general terminal anaesthesia. Any animals exhibiting signs of distress or significant ill health will be immediately humanely killed.

Why can’t you use animals that are less sentient?

Bone regeneration depends on the number of systemic factors. Interactions and dynamics working during regeneration are impossible to accurately replicate in invertebrates. Transgenic mice are currently the only system for analysing the developmentally regulated expression of mammalian genes in the complex environment that exists in the living organism.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In-vivo imaging will be carried out under terminal anaesthesia. This to minimise any stress or suffering of the animal still provide the required information to achieve the scientific objectives.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The dosages of drugs will be decided based on LASA guidelines. LASA guidelines will be used to guide surgery, including injection and surgery of mice for in-vivo imaging under terminal anaesthesia.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Attendance of internal and external NC3Rs seminars and conferences, in addition to monitoring of their website and signing up for the newsletter. We will also have access to an NC3R's regional manager as well as the establishments Named Information Officer

4. Understanding the Role of Nicotinamide Adenine Dinucleotide (Nad) Synthesis in Immune Cells

Project duration

3 years 0 months

Project purpose

- Basic research

Key words

Immunology, Infection, Metabolism, Therapy

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how Nicotinamide Adenine Dinucleotide (NAD) synthesis from vitamin B3 and its precursors supports immune cell function and underpins their role in infection responses.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Immune cells (white blood cells) importantly protect us from cancer and infectious disease. However, invasive fungal infections remain a significant global challenge that kill more people worldwide each year than breast cancer or malaria. For example, the fungal pathogen Cryptococcus neoformans, which is prevalent in the environment, causes infection mainly within the lungs and central nervous system (CNS) where it can lead to fungal meningitis and is responsible for >200,000 deaths per year. This problem is worsened by increasing rates of resistance to the limited selection of antifungal drugs available. It is therefore now becoming clear that improving immune responses to these

infections is urgently needed as a new approach that will help reduce the mortality rate for these infections.

In the last decade our understanding of how immune cells work has been transformed by the observation that their activity is very closely linked to a series of chemical processes known as metabolism. These processes break down nutrients such as sugar to provide energy and building blocks for the cell. Recent research has revealed that immune cells must dramatically change how they break down nutrients to carry out their protective functions. Additionally, studies in patients with infectious disease, such as chronic viral infection, have revealed that these processes are often impaired in immune cells in these conditions. This is likely also to be the case in fungal infection, although to date it has not been studied in depth. It may be possible therefore that correcting these changes in metabolism can restore normal, safe immune cell activity, and yield better treatment for these diseases.

This project will investigate one possible pathway to control immune cell metabolism and restore their normal function. Specifically, I will test how the production of a molecule called nicotinamide adenine dinucleotide (NAD) is linked to immune cell metabolism and their protective and harmful activity. NAD is produced from vitamin B3 and is required for all stages of the breakdown of sugar and other nutrients in cells. We have already identified that when immune cells are activated (switched on) in the laboratory, they turn on an enzymes which make NAD from vitamin B3. Additionally, when these enzymes are not active, immune cells have much lower rates of metabolism and produce much less of certain key immune signals. In this project we will alter NAD synthesis in immune cells in murine models of fungal infection (specifically Cryptococcus neoformans). This will help us to test whether new drugs targeting this might be helpful in these conditions.

What outputs do you think you will see at the end of this project?

We will generate new information on how NAD synthesis from vitamin B3 and its precursors support the metabolism and function of immune cell populations. We will identify whether targeting NAD synthesis alters immune cell activity and disease progression in murine models of fungal infection . Together these data will enable us to understand whether these pathways could be targeted as novel treatments for fungal infectious disease.

We envisage that this work will lead to several publications in reputable open access journals in the immunology/life sciences field.

We believe that the information generated over the project lifetime will support funding applications to begin human studies.

Who or what will benefit from these outputs, and how?

The project will deliver benefit to immunology and metabolism researchers in the short-term, through the generation of new knowledge and understanding about how vitamin B3 supports the metabolic activity of immune cells. It will also provide key information about how altering NAD synthesis in immune cells influences immune cell activity and disease progression in models of fungal infection.

In the medium term, the work here will be the first of its kind to interrogate the importance of NAD synthesis in the immune system and will identify specific components of this

pathway which could proceed to translational research and the design of novel therapeutic compounds.

In the longer-term, we envisage that this work may lead to the design and use of novel therapies which modulate NAD synthesis in the immune system to better treat infectious disease.

How will you look to maximise the outputs of this work?

To maximise the outputs of this work we have already identified two other groups within our establishment who work on similar models. These collaborations will enable sharing of expertise and optimisation of our experimental models. It will also maximise the chance of success of the project.

We will disseminate knowledge through publications, and for important data these may be released early into the public domain through non-peer reviewed pre-print servers such as bioRxiv. We will also publish data arising from unsuccessful approaches to avoid unnecessary repetition of these by other research groups.

We will promote our research through both internal and external presentations (e.g. seminars, conferences).

Species and numbers of animals expected to be used

- Mice: 5500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are used in these studies because: (i) the main components of their immune system are shared by humans; this is essential where complex immune responses as opposed to the function of individual genes or cells is being studied. Lower species provide an inadequate model of the immune system; (ii) a wide range of wild type and genetically manipulated strains of defined genetic makeup are available.

We will typically use adult mice for experiments, since by then the immune system is fully developed. The immune system undergoes substantial changes during development. Since we are interested in how the adult immune response responds to infectious disease it is therefore important to study this life stage.

Typically, what will be done to an animal used in your project?

Animals will be given vitamin B3 and/or its precursors to understand if these affect immune cell function. They will be given either in the diet or drinking water, or via short-term injection. Additionally, we will study the role of NAD synthesis in determining immune cell function during fungal infection.

Fungal infection will be modelled by infecting mice with Cryptococcus neoformans. This will be performed using well-established doses and routes (intranasal and intravenous), as well as well- defined experimental endpoints. The animals will be closely monitored and humanely killed when they reach defined end points. Our protocols and clinical monitoring criteria are designed to keep suffering to a minimum. These experiments will last for approximately 1-2 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

For injections, mice will experience momentary pain, which is expected to be mild and short-lived. Vitamin B3 precursor supplementation in the diet does not lead to adverse effects.

Cryptococcus infection model: Weight loss up to 15% is expected in 80-90% of wild-type animals in first 10 days of infection. Animals may develop other signs of infection, including ruffled fur (piloerection), hunched posture, and oculonasal discharge. These symptoms tend to be observed starting after 7 days of infection but may occur earlier in some strains. These experiments will typically last for 1-2 weeks.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect 75% of the animals in this licence to experience no greater than mild severity. The remaining 25% may experience moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Immune cells are part of a very changeable and complex biological system, which involves numerous cells that migrate around the body and interact with each other. Whilst simple in vitro models exist for investigating their behaviour in the lab, these do not always predict how immune cells may behave in the actual body.

In order to study immune cell responses that are relevant to human disease, we must use a species which shares all the major parts of the immune system. Among species that share the main components of the immune system with humans, mice are the least sentient option

Continued review of the scientific literature will be undertaken on a regular basis in order to identify any newly emerging technologies and models that could be potentially adopted in order to replace in vivo animal use.

Which non-animal alternatives did you consider for use in this project?

In vitro co-culture of distinct immune cell populations; in silico modeling of NAD synthetic enzyme activity in immune cell populations;

Why were they not suitable?

We will be undertaking in vitro experiments with human primary cells in parallel to those using animals. Wherever possible we will use these approaches to understand the mechanistic basis of our observations, employing molecular biology, imaging and immunological analyses of cells in culture.

However, the activity of the entire immune system during infection is highly complex, involving numerous cell types including the infected cells, cells of the central nervous system and diverse populations of immune cells, stromal cells and importantly vasculature. It is impossible to recreate these complex intercellular interactions in vitro or the vasculature, which is how immune cells enter and exit infected environments. Therefore understanding the complex consequences of targeting immune cell NAD synthesis within infection would not be possible.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We will use several different strains of mice to explore the importance of immune cell NAD synthesis from vitamin B3 in infection. Numbers have been calculated based on previous in-house data alongside pilot data and published literature.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have consulted the NC3R's Experimental Design Assistant tool during the experimental design phase. We will use an experimental approach which permits detection of differences, even when variables such as differences in sex and age are introduced. The approach controls for these variables by randomly assigning them to control and treatment groups. In this way we can maximise use of mice bred for the licence.

By using pilot experiments we will be able to determine the minimum number of mice to be used - typically we have determined that 6 mice per group will allow us to detect a reasonable treatment effect in most circumstances. However these calculations will be continually updated and improved throughout the life of the project.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

1. Pilot experiments

Small numbers of animals will be used in pilot experiments for each new type of study performed to assess intra- and inter-group variation. For most models in this project, optimal group sizes and variation are already well established in the scientific literature.

2. Inbred strains

The use of inbred strains (matched for age and gender) will reduce the amount of intra-group variation thus increasing the power of each experiment and reducing the group size needed to achieve statistical significance.

3. Sensible breeding of transgenic lines

Careful and considered approaches to colony management will be used to minimise wastage.

4. Multiple read-outs from same animal

Multiple read-outs taken post-mortem from experimental animals will further reduce the number of animals required. For example, a single animal can be used for the analysis of fungal burden, type and activity of immune cells present and analysis of diseased tissue for damage, metabolic activity and immune messenger molecules present.

5. Consistency of analysis

Where possible, the same person will perform procedures and carry out analyses. Alternatively, groups will be equally distributed between those performing this to avoid subject bias.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

1) We will use a mouse model with a mutation in vitamin B3 metabolism, this will allow us to study how immune cells synthesise NAD. The mouse model has no harmful phenotype.

2) We will use a model of cryptococcus neoformans infection to study how NAD synthesis from vitamin B3 supports immune cell protective function during this disease, which is a key objective of this research. This will involve intranasal or intravenous delivery

of the fungus, which will cause moderate, short-lived distress to the animal. In both cases the infection then proceeds to cause the mice to experience moderate effects, such as up to 15% loss of body weight. To study the immune response during this infection it is important that systemic infection and this level of severity occur. However, our protocols and clinical monitoring criteria are designed to keep suffering to a minimum. We will use Clinical Monitoring Sheets to ensure that unexpected adverse effects are identified as early as possible and that the severity limit is not exceeded. In most experiments the scientific endpoints are achieved prior to significant adverse effects occurring.

Why can't you use animals that are less sentient?

This work has to be conducted in mammalian species because the immune system, and its role in infection in mammals is much more complex than that seen in invertebrates or other vertebrates. Hence, studying rudimentary immune systems will not lead to a significant increase in our understanding of the regulation of the immune system in health and disease. The mouse is the worldwide standard laboratory animal model and its immune system has been the most intensely investigated, meaning that this information can be used inform future studies rather than having to repeat work in another species. This, in addition to the availability of genetically-altered mouse stains, allows much more rapid progress to be made. Finally, since the immune system changes substantially throughout development and life, it is critical to conduct the research in adult animals, whose immune system best reflects that of adult humans, who experience the diseases in question.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Injections will be given on alternate side to minimise discomfort for the animal and limit potential adverse effects that may occur by using the same injection site repeatedly.

LASA guidelines are used when determining volume to be injected for the various routes proposed and these will not be exceeded in this study.

Handling tubes will be routinely used to assess the animal's condition.

During the cryptococcus infection clinical monitoring will be carried out frequently to ensure that any unexpected adverse events are quickly identified and that severity limits are never exceeded.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will consult LASA guidelines on the handling and administration of substances.

ARRIVE and PREPARE guidelines will be used to ensure that the principles of good experimental design are applied, and subsequently reported, in order to reduce the number of animals required whilst ensuring that data is robust and reproducible.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly consult the NC3Rs website (www.nc3rs.org) and new staff will be directed to these web pages. Where new 3R advances occur we shall work with staff in our facility to adapt protocols or techniques. We will also seek advice and latest information through our Named Information Officer (NIO).

5. Modulating the Gut-Liver-Brain Axis for Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Diet, Brain, Microbiome, Gut-liver-brain axis, Ageing

Animal types	**Life stages**
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to further elucidate the interactive impact between lifestyle factors and the microbiome-gut-liver-brain axis in the context of metabolic disorders and its influence upon the progression cognitive decline. We aim to define novel strategies capable of delaying or preventing metabolic and brain disorders.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Debilitating neurological conditions such as Dementia, characteristically result in a decline in cognitive performance and represent an ever-increasing societal burden worldwide. In the absence of effective pharmacological treatment to prevent or reverse the underlying age-related and/or pathological mechanisms, it is necessary to shift focus towards preventative methods. With further development, lifestyle approaches offer a promising and novel route of mitigation. Increasing evidence highlights the importance of the microbiome and the gut-liver-brain axis upon modulation of brain functions. It is therefore important to undertake this work to further our mechanistic understanding of the microbiome- gut-liver-brain axis, particularly in the context of brain function. Understanding the underlying mechanisms will enable us to propose novel strategies to preserve brain health and prevent disease progression.

What outputs do you think you will see at the end of this project?

This program of work will strive to broaden our understanding of the role that lifestyle factors, gut microbiota and dietary patterns/nutrients play in the prevention of human brain ageing and the enhancement of a range of cognitive abilities. We anticipate this knowledge will enable us to develop novel strategies to delay and/or prevent age-related cognitive decline and pathological decline through the modulation of the microbiome and its communication with the host (gut-liver-brain axis).

It is anticipated we will continue generating high-impact publications in international peer-reviewed journals and to communicate our findings to colleagues in academia via invited talks to conferences and other academic institutions.

Finally, given the direct interest of the industry in finding solutions to improve cognitive heath, it is likely we will work with them to develop new products aiming at ameliorating cognitive performance.

Who or what will benefit from these outputs, and how?

The benefits of the proposed research may be considered both in terms of the potential clinical applications of our findings and the advancement of fundamental knowledge regarding the influence of diet, genetic and metabolic factors on cognitive functions in both normal and pathological ageing. In the short term, this project will provide a mechanistic understanding of the regulation of brain functions through dietary changes and the modulation of the gut microbiota. In the medium and long term, these studies will provide the background for new and promising preventive treatments for tackling cognitive decline and are aligned with a move towards the provision of personalised/stratified preventative strategies, particularly in individuals identified as being at “high-risk”.

How will you look to maximise the outputs of this work?

Output of this work will be published in high impact journals and presented at both National and International conferences. In addition, findings emanating from this research will be posted on social media (Twitter, LinkedIn, etc) which has an important coverage and reach.

We will also collaborate with researchers locally, nationally and internationally. Outputs will in form of high-impact journal publications and grant proposals to diverse funding bodies (UKRI, charities, industry).

Species and numbers of animals expected to be used

- Mice: 7410

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The interaction between the gut-microbiome, liver and brain (gut-liver-brain axis) has implications for numerous systems (e.g., metabolism, immune). It is therefore multifactorial and highly complex, meaning that that it cannot be fully nor precisely reproduced in vitro. For this reason, our research requires the use of mice in order to study such a system in its entirety.

In addition, the mouse models that will be used throughout this project will recapitulate different aspects of human disease and are thus indispensable to dissect the complex interactions occurring in the gut-liver-brain axis during specific processes of disease progression.

Typically, what will be done to an animal used in your project?

Typically, inflammation will be induced through administration of substances. Protocols involving the induction of inflammation will generally be short in duration ranging from a few hours up to a few days.

The route of administration will depend on the inducing agent, but this will generally be via injection or oral gavage. Procedures will generally induce only mild discomfort to the mice. Metabolic disturbances may also be established by maintaining animals on a particular diet such as a Western-type diet, or diets affecting gut-liver homeostasis (e.g high fructose diet).

Modulation of the microbiome will be achieved via administration of substances (e.g. antibiotics), live bacteria/bacterial products (e.g. probiotics, postbiotics) or food bioactives (e.g. fibers, flavonoids, PUFAs, etc.) and may be administered in combination with the different protocols proposed in this project. These substances will generally be administered orally by gavage on one or several occasions or integrated in food pellets or water.

Upon completion of experiments, mice will generally be humanely killed after blood extraction by cardiac puncture under terminal anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

The procedures described in this proposal involve transient and moderate stress or pain in well habituated animals, which will be controlled by use of anaesthesia and analgesic treatments where necessary. Doses of drugs used will be within published physiologically-active, non-toxic ranges and so no significant or frequent adverse health consequences are anticipated. Development of adverse health consequences following test substance administration are not expected. Animals will be routinely monitored for acute behavioural

signs of distress, developing changes in body weight (weight loss not to exceed 20% of pre-treatment level), changes in activity level or degree of grooming, ocular or nasal discharge, fever, hypothermia, or other signs of malaise. In the event of any complication or the development of any adverse reaction to the treatment, drug test procedure etc., experiment will be discontinued, and animals will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We anticipate that most of the mice will have a mild severity (90%; administration of substances, dietary challenges, microbiome modulation). 10% of mice may show a moderate side effect due to either surgery (minipumps) or dietary challenges affecting metabolism (western-type diet challenge).

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The interaction between the microbiome, gut-liver-brain axis, influences broad and complex systems such as immune and metabolic regulation which cannot be accurately modelled in vitro. for instance:

In vitro assays cannot model physiological and behavioural interactions.

Cell culture cannot fully replicate the features and interactions of brain cells apparent in vivo.

Bioavailability, tissue distribution and metabolism of compounds cannot be accurately modelled in cell culture.

Metabolic interactions cannot be modelled in cell culture systems because of the multiplicity of cell types.

Cognition cannot be recapitulated nor assessed in cell culture

In addition, a large proportion of the components of the microbiota cannot be cultured, supporting the need to perform our experiments in mice in vivo where we will analyse (specific components of) the microbiome and its influence upon the gut-liver-brain axis and the subsequent effect towards host metabolism and immunity.

The translational nature of our research requires testing microbe-based therapeutic strategies in mice prior to the translation into the clinical setting.

Which non-animal alternatives did you consider for use in this project?

Prior to embarking on animal experiments, as much evidence as possible will be collected in order to determine whether a candidate gene, food bioactives including their metabolites and microbes can influence brain function. Where possible, we intend to utilise brain slices and/or freshly dissociated neurons/glial cells obtained from animals, in order to determine the impact of treatment upon specific mechanistic events (e.g. synaptic plasticity and neuroinflammation)

When possible, we will also perform analyses in human samples obtained from patients/volunteers in collaboration with hospitals in the UK. These analyses in human tissue will complement our mechanistic studies using mice, furthering translational potential.

Why were they not suitable?

These in vitro and ex-vivo techniques are a useful tool to explore specific aspects of our research and will we use them accordingly.

However, none can fully replicate the complex metabolic and immune interactions of the microbiome, intestine, liver and brain in their entirety which is essential for our research.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Most of the procedures and protocols included in this project have been already used in our previous project license. These have generated meaningful and robust results that have been published in high impact factor peer-reviewed journals. Animal numbers are estimated on our current usage and projected proposed studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use several measures to reduce animal numbers.

The experiments are designed to use the minimum number of animals necessary. This includes the use of established immortalised cell lines as in vitro culture models for pre-screening studying basic mechanisms and ex vivo methods to analyse basic concepts of adult stem cell function and only bring the most likely candidates into in vivo studies.

We have calculated the number of animals for each experiment together with a statistician to use the minimum number of mice whilst still receiving meaningful and statistical significant results.

Time-lapse imaging enables serial and longitudinal data to be obtained from one animal over time rather than having to cull animals at predetermined times post treatment

Whenever possible the purchase of older animals will reduce animal use and need to breed and keep ageing/aged animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use homozygous mice in our breeding unless we are generating a new strain We use both male and female mice for our studies.
We isolate tissue from animals from experimental animals and store it appropriately for later use in histological and immunohistological analyses and/or share tissue when appropriate with collaborator and colleagues, thereby reducing the need to breed mice specifically for this purpose.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the standard species for transgenesis and are de facto standard for this work.

The choice of inflammatory stimuli is based upon published literature and their extensive use as a model of (neuro)inflammation. Dietary challenge experiments utilise various refined diets varying in the source and type of fat and carbohydrate content for which an extensive body of literature exists describing the conditions of their use and impact on metabolism and health including resistance vs. susceptibility to disease on which we can base our studies.

When chronic drug/dietary component administration is required, surgical implantation of osmotic minipumps will be preferred. Such technique also allows the direct delivery to the brain by using central cannulation.

The imaging protocols are highly sensitive allowing sequential visualisation of small numbers of animals without having to cull animals at predetermined times post treatment. The timing and number of imaging interventions is minimised through the adoption of established and published protocols using similar assessment and imaging equipment.

Wherever necessary local and general anaesthesia will be used to minimise animal suffering.

Why can't you use animals that are less sentient?

Our research requires whole bodily systems, including the gut-microbiome in order to understand complex physiological interactions in their entirety. The mouse is the least sentient animal that is both physiologically and genetically similar to human and allows both genetical and pharmaceutical manipulation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Welfare of the mice will be monitored after the administration of substances on multiple occasions by the experimenters and then inspected daily by qualified animal carers. Aseptic precautions will be taken to help prevent infections.

Surgical procedures will be carried out according to the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery. Analgesic agents will be administered as required after the surgery. In the event of post-operative complications, animals will be killed unless, in the opinion of the Named Veterinary Surgeon, such complications can be remedied promptly and successfully using no more than minor interventions.

Where the immune status of the animal might compromise health, they will be held in a barrier environment.

Other than in terminally anaesthetised animals dosing and samples procedures will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than a transient discomfort and no lasting harm. Typically, dose volumes will not exceed 20ml/kg for oral gavage, subcutaneous and intraperitoneal injection, topical application and 10ml/kg for intravenous injection. Strict asepsis is employed to prevent infection when a temporary cannula is set. In case hemorrhage, blockage of cannula or swelling around the cannulation site is observed the cannula will be removed.

Blood will be taken from superficial vessels. To avoid hypovolaemia or anaemia, typically not more than 10% of the total blood volume (TBV) will be withdrawn on any single occasion and typically no more than 15% TBV in any 28-day period.

Altered food composition should cause no adverse effects to the animal. Weight will be controlled weekly, and mice will be humanely killed if the weight gain restricts movement to reach food and water.

Animals will be humanely killed before signs of distress (eg loss of body weight, unkempt fur, abnormal posture, loss of muscle tone) become apparent.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All our studies follow the ARRIVE guidelines (provided by NC3R) which are widely accepted by the scientific community. In addition, we keep up-to-date by following updates provided by the Laboratory Animal Science Association (LASA) which, together with NC3R constantly publish improved and refined animal procedures.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All researchers involved in this project will be encouraged to subscribe to the NC3R newsletter and website and review current methods as soon as new guidelines become available. All researchers are also encouraged to subscribe to the Experimental Design Assistant provided by NC3R to design experiments the most efficient way and put stops if necessary.

6. Optimising Skeletal Muscle Structure and Function to Withstand the Challenges of Damage and Ageing

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Muscle, Regeneration, Sarcopenia, Ageing

Animal types	Life stages
Mice	adult, aged, juvenile
Rats	adult, aged, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to develop therapies to improve muscle mass and function in conditions where this is a problem, such as DMD or ageing.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The key benefits of this project are expected to be in the treatment of skeletal muscle that fails to work properly in humans. Results from this project will be beneficial in the future to

doctors as they are focused on human diseases, drug companies as well as researchers. Treatment of ill health will include patients with Duchenne Muscular Dystrophy which affects 1 in 5000 boys and leads to death at about 25 years of age. Skeletal muscle loss is found in many kidney diseases, cancers, and HIV infection. However, we are all likely to experience muscle wasting as it is the key feature of ageing which leads to it not only decreasing its size but also its ability to carry out its normal function. This process, called sarcopenia leads to a reduced quality of life and has huge costs associated with it to the economy. At present the only two regimes that are known to attenuate sarcopenia are exercise and improved diet. However, these two approaches are usually inappropriate for the large proportionate of the elderly due to changes in their physiology, hence, new approaches are required. Therefore, advances in developing therapies that reverse age-related muscle wasting are likely to be beneficial not only to those who suffer from it but also all of society since we all pay for their treatment through taxes. Money that can be saved based on our studies can be used to help others.

What outputs do you think you will see at the end of this project?

The key benefits of this project are expected to be in the treatment of ill health and the treatment of compromised tissue function in humans where skeletal muscle is affected. The immediate benefits will be the development of a range of chemical and molecular approaches to improve muscle function. This project aims to exploit our previous work carried out under a Home Office project licence in which we showed that muscle regeneration is greatly influenced by factors other than stem cells. This fundamentally changes the landscape in our understanding of the development of disease and most importantly the spectrum of people who can be treated. This is based on the flawed notion that stem cells were the key determinant of muscle regeneration. Therefore, people who have a deficit in stem cell number could not be treated. We show that this is not the case and so these people who would have been previously excluded from treatment can be considered as ‘treatable’.

The data coming out of this project will identify key molecular and biochemical processes that enable muscle to work efficiently. Findings from this work will be peer reviewed before they are published in internationally available journals. Ideally, we will publish in freely available journals that allows anyone to access our outputs. Furthermore, the outputs of the project will be presented to the scientific community at conferences where we can not only tell others of our work to potentially improve human health but to gain feedback on how to improve our work in the future. Another output could be the development of drugs and proteins that could be used for clinical purposes. We will also present our work at public meetings so that all members of society can hear about the outputs. This will be done at public lectures and at local Science Festivals which are held yearly.

Who or what will benefit from these outputs, and how?

The work carried out in the project can be used by many different groups in society.

The smallest group that will benefit from our work is the scientific community, the medical professionals and potentially the pharmaceutical industry. Studies outlined in the proposal will add to our understanding of the processes that control muscle growth and function. Several studies have identified means of increasing or decreasing muscle size. We have shown that the central idea of muscle biology that ‘big is better’ is wrong.

Secondly, we have shown that muscle regeneration is greatly influenced by factors other than stem cells. Importantly we have shown that changing how muscle uses oxygen can allow it to regenerate even when the muscle stem cells population has been halved.

This data will be of extreme value to our group in developing new projects, to other researchers in the university sector as well as pharmaceutical sectors as they are developing new drug targets.

The second group who will benefit from our research are those affected by specific diseases associated with muscle. Muscle wasting is a feature of many diseases in which it is either the primary affected tissue for example Duchenne Muscular Dystrophy which affects 1:5000 boys) or as an indirect target (e.g., chronic kidney disease). In many cases the outcome in either situation is fatal. It is also a process that is initiated following long periods of muscle disuse (for example bed rest after surgery), a process called muscle atrophy. Maintaining muscle mass is a key feature that not only improves the quality of human life but also decreases the chances of developing diseases. We suggest that our work will accelerate the development of therapies to treat muscle wasting disorders. Importantly we have shown that molecules including proteins can be introduced into disease models that promote muscle growth. These need to be optimised in terms of efficacy and safety.

The largest group who will benefit are the elderly as we hope to develop treatments for muscle loss that accompanies ageing.

Sarcopenia is defined as the degenerative loss of skeletal muscle mass and strength associated with the ageing process. According to the ‘European Innovation Partnership on Active and Healthy Ageing’, the number of people aged 65+ in the EU will increase from 85 million in 2008 to 151 million in 2060 with many older men and women suffering from mobility limitations that increase 1) the risk of injuries

and falls, 2) development of diseases and 3) increased demands on limited healthcare resources. Previous work has shown that skeletal muscle undergoes numerous changes with age including reduction in the size of muscle fibres. Results from work carried out under the previous licence showed that stem cells may not be a limiting factor in muscle regeneration in ageing and other parameters are even more important. We hope to use these discoveries to develop treatments that can be used to promote muscle health in the aged mice.

Lastly, this project will be relevant to a group of emerging diseases that afflict humans. One of the major implications of long-covid happens to be its impact on patients with COVID-19. Herein a large survey of data from around the world which assimilated the findings of over 1000 references found that patients with severe COVID-19 had decreased muscle function which impacted greatly on their quality of life. Therefore, there will be a need, possibly extending decades, for us to reverse this feature of COVID-19. Furthermore, there is extensive evidence that patients infected with the Zika virus also have major issue to do with their skeletal muscle with evidence for muscle necrosis as well as disruption of the muscle regeneration process.

The outputs of the body of work described in this application could be used by the pharmaceutical industry to develop drugs and proteins to slow the progression of muscle wasting in either the disease or the ageing setting or to reverse this process.

The medical, pharmaceutical industry and public will benefit our work in the medium to long term (either towards the end of the timeframe of the licences and afterwards) since a large number of other studies (safety testing, clinical trials etc) are necessary before they can be translated into treatment.

Additionally, the results will be used by other researchers to refine their work since we will make advances on which they can build. Our work will mean that others will not have to conduct similar experiments and based on our work they can refine their studies which should translate into reduction in number of animals used for experimental studies. This group will benefit from our work during the lifetime of the licence.

How will you look to maximise the outputs of this work?

All outputs of this project will be communicated through the most appropriate channels. This will include the publication of results in internationally peer reviewed journals. Where possible we will endeavour to publish in open access journals so as facilitating dissemination of findings. Furthermore, all research outcomes based on this licence be it successful or unsuccessful will be made available to any user upon request. In terms of timelines, outputs and data will be made immediately available upon request for information.

Species and numbers of animals expected to be used

- Mice: 5000
- Rats: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice and rats will be used in this project, as they can be used to develop the symptoms of the diseases that are the focus of this project: muscle wasting associated with disuse, changes in diet, regeneration. Mice and rats at all stages of post-natal life will be used. Genetic strains of mice and rats will vary depending on the nature of the gene modification in which they were carried out.

Typically, what will be done to an animal used in your project?

Typically, mice and rats will be bred so that they have the correct genetic mutation, which develop a phenotype in skeletal muscle.

Some animals may be placed upon an adjusted diet to induce a muscle phenotype.

Thereafter they will be treated with a regime, including substance administration, which is intended to have an outcome on muscle structure and function.

The animals may undergo physiological examination, for example determining how long they can exercise, which will allow us to gauge how well the muscles are working.

Thereafter the animals will be killed, and tissues collected for further investigation aimed at revealing how the treatment has altered the function of skeletal muscle.

What are the expected impacts and/or adverse effects for the animals during your project?

It is likely that only a minor number of animals undergo procedures in this licence that experience pain or show loss of body weight (dietary restriction and starvation). We plan to terminate the experiment when the intervention to induce tissue wasting results in greater than 15% of body weight loss compared to control rodents.

Some animals will undergo surgery and anaesthesia in order to course tendon and nerve transections, as well as minipump insertion, which will require regimes to control pain. Pain issue is resolved usually in a matter of one to two days.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The most severe category is moderate which will be experienced by no more than 20% of the animals in this proposal.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Skeletal muscle is a complex tissue made of not only muscle fibres but also blood vessels and nerves. The health of muscle is dependant of not only on features of the muscle fibre, but also on input from surrounding tissue, especially the nerves and blood vessels. These tissues all respond to changes in their surroundings, for example the food that we eat, in a way that we are only beginning to understand.

Although we can grow cells in a test tube, these do not behave in the same way as muscle in an animal. Since these test tube models lack influence from anything other skeletal muscle, they poorly represent which actually occurs in the human body. Therefore, it is still necessary for work on mice and rats that is outlined in this proposal.

Which non-animal alternatives did you consider for use in this project?

With some aspects of the project, experimental animals can be replaced by in-vitro testing, and as outlined in the programme of work such in-vitro testing will replace intact animal experimentation wherever possible in the project. In vitro experiments especially using skeletal muscle cell lines (thereby eliminating the need to sacrifice mice and rats for the

isolation of experimental material) will be used as a matter of choice at the start of any applicable study. For example, cells will be used to assess muscle damaging reagents to gain information about which stage such molecules may impact on the tissue found in the body. At present there is a huge amount of excitement regarding the use of organ culture protocols to understand the workings of a complex tissues. At present, the organ culture protocols specifically for skeletal muscle are at development phase and not as advanced as other systems for example the gut. This is undoubtedly due to the cellular nature of skeletal muscle (the muscle fibre) and its need for complex interactions with both other cells (blood vessels and nerves) as well as relatively undefined extracellular components including the matrix. Therefore, at present it is a necessity to use animal models to investigate skeletal muscle. However, it is inevitable that organoids for skeletal muscle will be developed and we will use these at the first given opportunity if they are a good substitute for animal studies.

Why were they not suitable?

While in-vitro studies are very informative on the nature of skeletal muscle. Skeletal muscle can be induced to differentiate and grow into myotubes, but these are no way near the level of maturity seen in a mammal. They are structurally and functionally very different to myofibers which constitute the cell unit of adult skeletal muscle. For example, they lack the neuromuscular junction as well as the myotendinous junctions which are critical to the normal workings of skeletal muscle.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For quantitative experiments sample size will be set statistical predictions based on the difference we want to detect to give us an indication that a treatment is working. We will draw on our work conducted using the same animal models and how the experiments were run to give similar but unique outcomes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

More generally, advice will be sought from our Consultant Statistician, where appropriate, to ensure a statistical design that is used is efficient and minimises the number of animals required yet maintains sufficient precision and power. For example, advice will be sought when a specific pre-clinical model introduces large subject-to-subject variation.

Furthermore, our studies have shown that the impact of muscle growth promoting molecules effects more than just the primary tissue under investigation, that being skeletal muscle. Therefore, for an intervention to be examined further for possible use in humans, it is now apparent that an intervention should be examine at the organismal level as well as a range of tissues. We have developed a pipeline that extracts the maximal amount of data from each animal by not only determining its behaviour and movement prior to its

death, but then to isolate as many tissues as possible. This approach means that a single animal will generate data related to a wide variety of bodily functions.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The proposal aims to minimize animal usage by drawing on our extensive experience in this area of research. These insights will be deployed not only to keep the number of mice to their lowest possible number but also to conduct the experiments in the shortest time span thus minimizing animal suffering.

We will in all cases initiate experimentation with novel substances by examining their impact on muscle structure and function on a small number of mice or rats (2) before moving on to larger scale studies assuming that no adverse effects manifested.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The choice of models employed in the project reflect the physiology, structure, and regeneration properties of humans, which is critical since this project aims to develop treatments focusing on health. Where possible the use a genetically modified mice or rats will be used to remove the need for surgical or chemical interventions in establishing a muscle phenotype, thus reducing the numbers of animals experiencing pain, suffering and distress.

Rodents have been extensively used to make major breakthrough in our understanding of human skeletal muscle biology since they are mammals, and their tissue architecture resembles that of us. The mouse is particularly attractive as an experimental model as a huge number of genetically modified lines have been generated which allow us to hone into the function of specific genes in the muscle development program. A limitation of the mouse model is that related to the cellular structure in comparison to humans, particularly the composition of muscle fibres. Herein the rat offers significant advantages over the mouse in that its muscle fibre profile is more similar to the human than that of the mouse.

Why can’t you use animals that are less sentient?

Small rodents (mouse: rat) are the simplest appropriate pre-clinical models to study these diseases and their potential amelioration. While there are species differences in physiology between humans and rodents, these are minimal and the use of species genetically closer to humans is not required by the appropriate regulatory bodies and thus is not proposed as part of this project. The mouse is the species of choice due to availability of appropriate genetically modified lines that phenotypically display accelerated ageing. Furthermore,

genetically modified mice, either through gene knock-out or gene over-expression studies allows us to develop mechanistic explanations at the molecular level of the processes of muscle growth. Rats are also appropriate since they have muscle structure which differs from mice (as they are metabolically more similar to humans than mice) and in certain aspects mimics the human condition.

Non-mammalian models including nematode worms and fruit flies do not share the same physiological parameters as humans and are thus not informative for this type of study. Zebrafish are not a relevant model for this project as their muscle structure and functions differ greatly from mammals. For example muscles tend to be composed of single celled fibres in contrast to the human syncytial fibre which is made up of tens of thousands of nuclei.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals may experience stress when undergoing behavioural and exercise tolerance regimes. This will be minimised through a protocol of familiarisation in which the regime is introduced in a stepwise process. Other refinements to decrease harms to the animals will through constantly monitoring to pick up signs of adverse effects during and after any intervention followed by immediate post-operative care and pain management.

Rodents lose a huge amount of body mass following total food deprivation undoubtedly due to their high metabolic rate which can rapidly develop into torpor. We have carried out work over the past 8 years to investigate the impact of starvation on skeletal muscle function and have concluded that food deprivation for a period of 24 hours is adequate for us to reach major outcomes on this tissue rather than as previously maintaining animals on this regime for 48 hours.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will seek to carry out the experiments using best practice advised by differing parties including:

Laboratory Animal Science Association (LASA) for best practice guidelines for administration of substances and genetically modified mouse welfare guidelines.

We will also turn to the Workman guidelines for cancer research (https://www.nature.com/articles/6605642) as these principles are particularly relevant to our study as they describe welfare issues and how to use best practice for models which experience tissue wasting, the core focus of this proposal. Please see:

Workman P, Balmain A, Hickman JA, McNally NJ, Rohas AM, Mitchison NA, Pierrepoint CG, Raymond R, Rowlatt C, Stephens TC, Wallace J (1988) UKCCCR guidelines for the welfare of animals in experimental neoplasia. Br J Cancer 58: 109–113

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will seek advice from the named veterinary surgeon for alternative approaches using either less sentient animals or non-animal systems that allow us to achieve our goals.

Furthermore, we will maintain our knowledge and best practice of 3Rs by attending relevant conferences and meetings as well as referring to appropriate websites, such as NC3Rs.

7. Characterization of Novel Antimicrobial Agents and Treatments

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

infection, antibiotic, antifungal, antiviral, vaccine

Animal types	Life stages
Mice	adult, juvenile, neonate
Rats	adult, juvenile, neonate
Cotton Rats	adult
Guinea pigs	adult
Hamsters (Syrian) (Mesocricetus auratus)	adult,

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This aim of this project is to provide in vivo support services to pharmaceutical and biotechnology companies and academic establishments to support the development of antibacterial, antiviral and antifungal treatments and vaccines.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Although there have been tremendous advances in the treatment of infectious diseases there are still many developing and unmet clinical needs.

Antibiotic resistance has been described as a slow pandemic, with the UN estimating up to 10 million deaths a year globally by 2050 unless new treatments are developed as a matter of urgency. As antibiotic drugs support many aspects of modern human and veterinary medicine such as cancer treatments, cystic fibrosis treatment and surgical procedures this has the potential to impact all aspects of society.

In addition to antibiotics, there is a growing resistance problem with resistance to antifungal drugs, causing a significant rise in the number of deaths, particularly in clinically vulnerable groups such as pre-term babies, HIV infected and other immunosuppressed patients. There are estimated to be above

1.7 million deaths globally due to fungal infections.

Although a lot of progress has been made with the development of antiviral drugs, there is still huge unmet medical need. The ongoing SARS-CoV-2 pandemic has illustrated the need for having a range of antiviral drugs available targeting virus families considered to be a risk of causing the next pandemic as well as being able to rapidly develop new drugs when required. There are also established infections such as hepatitis C, influenza viruses and herpesviruses that are killing patients where no effective antiviral treatment is available. Worryingly in recent years antiviral drug resistance has started to develop in HIV infected populations, which is a significant public health threat.

For all three classes of infectious agents vaccines have a critical role to play in the prevention and increasingly treatment of these infections. As with drug treatment, there is still significant unmet need for new and improved vaccines for a wide range of disease such as tuberculosis, hepatitis C and HIV.

The work conducted under this project licence is critical to supporting the early stages of developing new treatments to these three global public health threats.

What outputs do you think you will see at the end of this project?

The studies performed under this licence will contribute to the early stages of drug development projects

This will build on studies performed not using animals where potentially useful treatments are initially identified. The animal studies will provide information on whether a potential treatment or vaccine is effective in a complex organism with similar biological processes to

humans. This will help project teams understand how to improve their treatment, whether their approach to the drug target is not effective in an animal model, or, ideally, provide evidence of efficacy so the treatment or vaccine can be progressed towards safety assessment and human trials.

Where significant results are not subject to confidentiality agreements they will be communicated more widely at scientific meetings or published in peer reviewed journals.

Who or what will benefit from these outputs, and how?

In the short term clients, project teams and funding bodies will be able to make progression decisions to be able to better target their limited financial resources on to the potential treatments with the highest likelihood of clinical success.

In the medium term stopping the progression of projects with little chance of success (either due to lack of relevance of the target or the inability to develop a potential medicine that has a clear beneficial effect in animal models) will also mean that animals will not be used in safety assessment and human volunteers will not be put at risk in clinical trials.

In the long term the successful identification of potential treatments will result in reductions in patient mortality, improved clinical outcomes (including shorter hospital stays) and reduced societal costs.

How will you look to maximise the outputs of this work?

Where there may be broader interest in an animal modelling approach, and if the studies are not subject to confidentiality agreements, these will be published or shared at relevant conferences. Refinements to techniques will be shared with others working in the field via individual contacts made by the establishment Named Persons

Clients and collaborating partners will be encouraged to publish all results in journals or share at relevant conferences.

Species and numbers of animals expected to be used

- Mice: 26,000
- Rats: 4500
- Guinea pigs: 100
- Hamsters (Syrian) (Mesocricetus auratus): 1,000
- Other rodents: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

These animal studies serve as a bridge between computer or cell-based experiments and human clinical trials. These studies will, in many cases, be the final studies investigating the likelihood of a potential medicine having a benefit before clinical trials are started.

In the majority of cases adult animals will be used as these are representative of the majority of the patient population. Rarely, where a specific infection or treatment focusses on the paediatric population, juvenile or neonatal animals may be used.

Most animals used in this project will be mice (occasionally including genetically altered animals) as these have been demonstrated to provide information that enables decisions to be made on the progression of treatments. Their reactions to infections and response to antimicrobial treatments have shown good alignment with those seen in human patients.

Using genetically altered animals allows the investigation of human-specific targets and treatments for these targets.

Other species will be used where a specific infection cannot use mice such as investigating skin infections in rats, gastrointestinal disease in hamsters, surgical implant infections in guinea pigs and respiratory infections in cotton rats

Typically, what will be done to an animal used in your project?

Most studies undertaken will be of short duration, lasting less than 7 days.

Animals will typically (approximately 70% of studies) have two injections of a drug to reduce the immune response, then be infected with the infectious organism of interest. Most infections are performed under general anaesthesia to minimise the impact on the animals.

The commonly used infection routes will be into the thigh (to model soft tissue infections), lung (to model pneumonia) or into the blood stream (to model infections that spread throughout the body).

With the exception of vaccination studies, all treatments in these models will only start once the infection has been established. When investigating antibiotics, the animals will normally be treated with the potential treatment several times a day, with studies usually being completed within 5 to 24 hours of infection. This may require the implantation of catheters into blood vessels to allow the more frequent treatment (up to once per hour, or even continuous infusion) to be performed with less pain and distress to the animals. For fungal or viral infections the study may run over several days, but frequency of treatment administration will be reduced, usually no more than 4 times daily for up to 7 days.

Other routes of infection may occasionally be used such as skin, bladder, vagina, bone or intestine to model very specific clinical conditions. In these cases animals will generally be treated less frequently, usually no more than 4 times daily for up to 7 days, but

occasionally may require surgical implantation of catheters into blood vessels to allow for infusion of potential treatments when this is the likely route of treatment in patients.

When investigating treatments for infected surgical implants, the animals are anaesthetized and a small piece of surgical implant material (usually a length of catheter tubing) is placed under the skin. This may already be infected at the time of surgery or the wound may be allowed to heal and then an organism injected into the implant to cause the infection. These animals will be left for several days for the infection to develop then treatment started, usually no more than 4 times daily for up to 7 days.

In small numbers of studies investigating potential antimicrobial treatments blood samples will be taken to measure the blood levels of the treatment or other markers of effectiveness of treatment.

Studies investigating vaccines will usually last up to 6 weeks and the animal will be dosed with the potential vaccine on up to 3 occasions 14 days apart. Initial studies will only require blood samples to be taken to measure the response to the vaccine, but a small number of studies will be performed using vaccine candidates that have produced a robust response where the animals are vaccinated and then infected to determine whether the immune response seen is protective against the infection.

All animals will be closely monitored according to the expected progress of the infection and will be removed from study if they reach the humane end point. Where infections are considered to be painful (for example thigh and bone), pain relieving drugs will be given to the animals, with response to the pain relief assessed as part of the regular monitoring.

At the end of the studies the animals are humanely killed and tissues taken for assessment of the levels of infection in the tissues of interest.

What are the expected impacts and/or adverse effects for the animals during your project?

The most significant impacts on the animals will be a result of the infections. In general infections will cause loss of appetite and so weight loss, reduced activity and changes in core body temperature.

With thigh, bone and systemic infections, pain may also be seen With lung infections an increase is breathing rate and changes in breathing pattern may also be seen. In bacterial models these will be towards the end of a study, usually no more than 24 hours after infection. For fungal and viral models these signs will also occur in the final hours prior the end of the study which may be up to one week in duration

Administration of substances causes brief stress and pain due to handling and needle insertion. These are controlled by skilled handling and minimising the numbers of administration and sampling events.

Some genetic alterations can cause adverse effects which are very dependent on the gene. We plan to only use genetically altered animals that have Mild adverse effects.

Anaesthesia for infection and treatment administration can result in heat loss and short term unpleasant experiences when recovering. Animals will be closely monitored, have heat supplied throughout the infection process and only undergo the minimum number of anaesthetics required to give a satisfactory scientific output.

Surgical cannulation of blood vessels will cause pain that is controlled by the use of pain-relieving drugs: generally this lasts for 48 hours, but the animals are closely monitored in case further doses are required.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice - 50% Mild and 50% Moderate

Rats - 50% Mild and 50% Moderate

Hamsters - 20% Mild and 80% Moderate

Cotton rat -30% Mild and 70% Moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Many of the initial studies performed to help in the validation of antimicrobial targets and assessment of potential medicines now take place in computer, genomic, receptor and cell-based experiments.

The hollowfibre infection model can give an intial assessment of how the antibacterial treatments may behave in a living organism prior to being used in clinical trials.

However, even with these advances, computer and cell-based approaches still do not allow the response of the infectious agent to be assessed in a way that reflects the full

complexity of an integrated mammalian system, particularly where the immune system response is a significant part of the expected action of the treatment.

This project aims to provide the data from a complex, integrated organism to allow decisions to be made on whether to progress potential anti-infective medicines or vaccines to the next stage of development.

The use of non-mammalian species such as the waxmoth larva infection model is also seen as a potential replacement approach, and useful information is be obtained from these approaches: however due to the degree of differences at a genetic and molecular level these models are more suited to very early scientific investigations rather than drug discovery and development.

Which non-animal alternatives did you consider for use in this project?

Some or all of computer modelling, genomic, cell-based and non-protected animal approaches will be used prior to undertaking studies on protected animals to minimise the number of studies and impact on the animals.

Clients will be asked to provide information on the work undertaken with approaches not using protected animals and an outline of literature reviewed searching for alternative approaches prior to performing animal studies. It would be expected that molecular and cell-based assays will have been undertaken to have confidence that the potential anti-infective medicines or vaccines is likely to be effective in the animal model. These studies will usually include investigations on the ability to affect the infectious agent, assessing the properties of potential medicines for biological availability and likelihood of reaching tissues of interest and looking for early indications of toxicity liabilities. These test will reduce the numbers of experiments performed and increase the likelihood of those completed to deliver meaningful results.

Why were they not suitable?

There are no non-animal alternatives that can currently replicate the full complexity of an infection in the mammalian body. Now and for the foreseeable future there will need to be animal experiments performed to perform initial investigations into the efficacy of potential anti-infective medicines or vaccines as part of the process of bridging from non-animals studies to clinical trials.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated numbers are based upon the number of animals used in the preceding project licence, which reflect the scientific and commercial demand for assessment of potential antimicrobial treatments, with an adjustment to reflect that studies using species such as cotton rats and guinea pigs have significantly reduced.

As there is an increased focus on anti-microbial resistance in relation to public health, it is not anticipated that there will be a significant reduction in the overall number of studies being performed in this therapeutic area.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used pre-existing internal data from ongoing models to calculate group sizes that are likely to provide a robust experimental output without using excess animals. These calculations are normally performed using commercially available statistical software (currently StatsDirect).

The minimum number of appropriate control groups and control animals are used, however these are critical for providing robust data so there is limited opportunity to reduce these further. On occasion it is possible to use shared control groups when performing multiple studies with the same infectious agent on the same day.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Wherever necessary pilot studies will be performed (under separate licence authority) to help design the decision-making studies, including identifying the dose level of infectious agent that gives the best chance of identifying a clinically relevant effect with the minimum possible adverse effects.

Prior to infection studies, investigations will be performed to determine whether the anti-infective treatment is tolerated and if possible also how it is absorbed and removed from the body. This allows modelling to be used to predict the doses required for the infection studies.

In some studies tissue samples will be taken after the study is completed to measure how the treatment has moved around the body, and may help prevent further studies being performed should unexpected results be found.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically altered animals that demonstrate no or only Mild signs will be used.

The majority of studies in this project will be using infection models which reflect conditions seen in the clinical situation. All these studies are designed to have the shortest duration and lowest doses of infectious agent that allow for effectiveness of treatments to be assessed. The severity experienced by the animals is managed with observations at a frequency appropriate for the model, including multiple assessments overnights for more rapidly progressing situations such as the sepsis models.

The use of pain relieving treatments and agreed humane endpoints helps to limit the adverse effects experienced by the animals

The models include:

- Sepsis (blood borne infection),
- Foreign body (usually associated with surgical implants)
- Urinary tract infections (bladder and kidney)
- Vaginal
- Bone
- Skin and skin structures
- Abscess and soft tissues (thigh)
- Lung and respiratory tract
- Clostridium difficile (also known as Clostridioides)
- Gastrointestinal infections (usually Salmonella)

One additional models is the investigation of response to vaccine candidates, which will have severity minimised by careful choice of doses and the use of pain-relief medications to reduce any adverse effects that may occur.

Why can’t you use animals that are less sentient?

Prior to performing procedures under the authority of this licence, some or all of computer simulation, simple receptor or cell-based studies, complex cell-based systems (e.g. organ on a chip) or simple animals (waxmoth larvae) will have been performed to focus on potential medicines that have the highest chance of success.

These simple models do not provide sufficient data to enable the development of a thorough understanding of the effects of the potential anti-infective in a fully-integrated

organism, which allows the complex interaction of the treatment with many body systems to be assessed prior to moving on the human clinical studies.

Most studies will be investigating responses that develop over many hours or days so the use of terminal anaesthesia is not appropriate.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

On site, working proactively with the AWERB, NVS and NACWO, there is a culture of constant improvement to animal care, control of adverse effects, performance of procedures and study design.

Scoring systems are used to identify early intervention and end points in studies, pain-relieving drugs are used when there is concern an animal is suffering, and monitoring is performed as often as required, including throughout the night.

Further refinement to technical procedures and housing are implemented when they are shown to be beneficial for the animals and will not reduce the quality of the scientific outputs.

When an established model is identified from the scientific literature and proposed for use under this licence, a small number of pilot studies will be performed under a separate licence authority to ensure the model delivers high quality scientific data whilst allowing the development of adverse event controls and scoring systems that may be able to be used to reduce the severity experienced by the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In addition to Home Office and EU guidance documents, relevant best practice guidance will be sourced from the NC3Rs (e.g. ARRIVE Guidelines, blood sampling, experimental design), NORECOPA (e.g. PREPARE guidelines), LASA (e.g. blood sampling, drug administration, aseptic surgery) RSPCA (e.g. septic shock model guidelines) and model-specific publications.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

On a monthly basis the PPL Holder receives and reviews automated literature alerts on animal models and journals relevant to the project licence.

When a new infection type or specific animal model is proposed, a thorough literature review is performed to determine the most scientifically relevant approach whilst causing the least harm, and a new automated alert generated.

In addition to conference attendance, webinars and discussion groups are participated in by the PPL Holder and scientific staff.

8. Genetic and Cellular Mechanisms of Kidney Development and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

polycystic kidney disease, genetic modifiers, disease progression, treatment, rare disease

Animal types	Life stages
Mice	adult, pregnant, neonate, juvenile, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to determine the role of genetic modifiers in Autosomal Recessive Polycystic Kidney Disease (ARPKD). The project also aims to identify potential genetic and pharmacological treatments that could impact disease progression and treatment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Rare diseases affect 1 in 2,000 people and with at least 8,000 known rare conditions, they impact millions of individuals worldwide. Autosomal Recessive Polycystic Kidney Disease (ARPKD) is one such condition that mostly affects individuals from early on in life, causing massive stress not only to the patients, but their families. To this day, no pharmacological cure exists and the individuals' only option in many cases is a kidney and/or liver transplant. ARPKD is caused by changes in DNA in PKHD1 and DZIP1L1. Patients with the disease display symptoms such as enlarged kidneys; liver defects and smaller lungs, with death in around 30% of affected neonates. A range of disease severity is observed in ARPKD, with some patients dying in the first 5 years of life, whilst others maintain kidney function until their early 20s.

This project will shed light on the molecular mechanisms of ARPKD and conduct pre-clinical trials on appropriate mouse models to identify promising treatments.

What outputs do you think you will see at the end of this project?

At the end of this project, we will have acquired a better understanding of the role of various genes in ARPKD and this knowledge could inform patient diagnosis and prognosis. It is likely that at least one of these mouse lines will help with the identification of novel genes that modify polycystic kidney disease severity; this could help us with predicting disease prognosis. Furthermore, once fully characterised, some of these lines could be used for pre-clinical trials on compounds that slow down polycystic kidney disease progression. We will also test at least two potential repair factors on mice and be able to determine if they may be of therapeutic value in ARPKD. We hope to find that at least one of them slows down kidney disease, so that future clinical trials assessing the efficacy of this factor can take place. We will publish all the information we have acquired from this project for both specialist and non- specialist audiences and communicate our findings at research conferences and patient information days.

Who or what will benefit from these outputs, and how?

The short-term benefits will be increased knowledge accumulation on the molecular mechanisms of ARPKD. Where genes that are linked to ARPKD are identified, these could be in the future added to genetic screening profiles to aid with diagnosis and prognosis of patients.

Appropriate mouse models will be made available to researchers for the development and testing of targeted treatments.

Any effective repair factors tested and found to be effective, could go on to be tested in clinical trials.

We will communicate our findings to non-specialist audiences, eg. patient families, so as to update them on the research progress and investigate whether their experiences as patients can inform our further work. At the end of the project, our research outputs will be accessible to researchers, clinicians and policy makers and if proven that additional genes impact ARPKD, this would be beneficial to diagnosis, prognosis and treatment.

How will you look to maximise the outputs of this work?

The outputs of this work will be communicated to my current kidney and cilia networks via the Ciliopathy researchers' network and the British and European Renal Associations. We will expand our current network of research collaborations in kidney, cilia and lung to include specialists in liver physiology and to create a global network of multi-organ ARPKD knowledge. Any unsuccessful approaches or negative data will be published to relevant open access scientific publications e.g F1000research, so as to avoid research repetition and inform future research directions.

Species and numbers of animals expected to be used

- Mice: 10,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our work will be exploring the role of genetic modifiers in ARPKD severity in mice. We will be investigating the effect of genes and environmental factors on organ function during development and in paediatric and adult stages, so as to assess ARPKD manifestation and severity at all stages of the disease and thus acquire a thorough understanding of the role of various factors that affect organ structure and function throughout the whole life cycle. Mice are chosen as a species of interest as they have a high genetic similarity to humans and are of crucial importance for pre-clinical trials. Mice have been successfully used in the past to model human Polycystic Kidney Disease (PKD) and the use of a mouse model led to the discovery of tolvaptan, the only drug currently available for dominant PKD. Other animals such as zebrafish or flies are not appropriate for PKD research, as they do not fully mimic the symptoms of the disease.

Typically, what will be done to an animal used in your project?

Already existent genetically engineered mice will be placed in metabolic cages for 24 hours to investigate their kidney function. They will also be exposed to a number of environmental factors for short periods e.g. nephrotoxic agents, repair factors to investigate if they modify organ function.

Factors will be administered orally or by intraperitoneal injection to postnatal or adult mice, for a maximum of four times over a 28-day period. Mice will also be assessed for serum markers of kidney function and they will be video monitored as well as in vivo imaged to investigate how their genetic modifications and interventions by repair and damage agents affect their kidney and other organ function and overall well-being. All mice will be humanely killed at the end of the procedures.

What are the expected impacts and/or adverse effects for the animals during your project?

None of the procedures used should cause lasting harm. Some stress may be associated with the procedures that will be performed e.g. metabolic cage housing, tail bleeding and mice will be closely monitored and appropriate interventions will be applied, should this arise. It is also possible that some mice may develop polycystic kidneys. If this were to occur, their diet will be carefully monitored and daily well-being monitoring will take place. Some of the substances we administer may result in some loss of appetite and weight loss or weight gain, polyuria or polydipsia, however, these symptoms are transient. We will closely monitor mice for signs of ill health such as piloerection, hunched posture, inactivity or inappetence. If any such symptoms are observed, mice will be monitored more frequently. Should the signs persist within a working day, the animal will be humanely killed. In addition, mice will be weighed daily and any animal that loses 15% of its starting body weight will be culled.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Around 80% of animals will experience mild severity or sub-threshold severity, where animals are bred, genotyped and then killed to obtain tissues and body fluids. Of the 20% of mice remaining, 10% of these mice (used in Protocol 3) could reach moderate severity. A further 10% of the total mice will be used in Protocol 2, however, only one quarter of these mice could reach a moderate severity limit i.e. only those where the gene of interest has been completely removed (homozygotes) therefore the total percent of mice that may reach moderate severity is 10% from protocol 3 plus 2.5% from protocol 2= 12.5%, i.e. 450 mice.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

As the kidney is a complex organ which helps maintain a healthy digestive and circulatory system, it is not possible to undertake certain comprehensive physiological studies outside the context of an intact organism where all native cell and tissue types are present in their normal numbers and arrangements (e.g. epithelium, mesenchyme, vasculature, lymphatics). In addition to all of these components being present in their normal arrangements (morphology) constituting an entire organ, the kidneys must be connected to an active circulatory system, so as to be able to perform their normal physiological function of removing waste from the body. There is currently no alternative to a whole animal that can reproduce all of these factors together.

Another key reason why we cannot currently entirely replace animal use for this project is that this study focuses on how developmental pathways and their disruption have a long-lasting effect on adult health and disease suffered in the postnatal animal. It is therefore necessary to perform these studies on mice with developmental disorders maturing into effected adults. Moreover, we wish to identify genes that are required to establish normal structure of the whole kidney, e.g. for the kidneys to form their full complement of sub-cellular structures. We do this by analysing the whole kidney both during development and post-natally, if embryonic development is affected, in the context of a mouse carrying a genetic modification in a gene(s) of interest. This type of analysis must be undertaken in whole intact kidneys that have developed in a genetically modified in vivo environment, so that it is not based by epithelial cell types that are predominant in cell lines or kidney on a Chip.

Which non-animal alternatives did you consider for use in this project?

Whenever possible we make use of non-animal alternatives. We have explored the role of genetic modifiers in mouse and human cell lines e.g. kidney epithelial cells. This project is based on results from human kidneys from ARPKD patients obtained upon nephrectomy, but since this is a rare disease, numbers are very limited. Our work on mouse and human cell lines and human ARPKD kidneys has identified specific genes that are dysregulated in ARPKD. These are the genes that we will be further investigating in mouse lines. Throughout the project, where possible, non-animal experiments will be employed e.g. human tissues samples, kidney epithelial cell lines and we will consult various databases of existing human and mouse information. We will monitor for developments in the field and the potential application of non-animal alternatives through the British Renal Association, the European Renal Association, peer-reviewed published work in nephrology journals and nephrology conferences' presentations.

Why were they not suitable?

In vitro experiments do not allow us to study the parallel effect of genetic modifiers in a whole organism, which is crucial for a disease such as polycystic kidney disease, which besides the kidney may also manifest in the liver, pancreas and lungs. Parallel studying the molecular mechanisms of disease manifestation in all these organs may shed more light in disease prognosis and treatment. Human kidneys from ARPKD patients are extremely rare, very hard to find and difficult to genetically modify. Studies on post mortem tissue do not allow us to investigate the physiological function of the kidney. Kidney on a ChIP is not appropriate for this work, as it mainly permits of the growth of one specific kidney cell type and this is not the cell type that is affected in ARPKD.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals is estimated based on previous experience of the number and type of experiments we will do over the duration of the project. E.g. in a typical experiment described in protocol 3, 32 mice are required for a backcross and 50 mice for the intercross, as per our previous experience.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Where we had previous data from similar experiments available, online power equation calculators were used to determine animal group sizes required to obtain statistically meaningful data. We also employed the NC3R's experimental design assistant where possible to ensure that animal numbers are the minimum required to obtain meaningful data.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continue to make use of databases of human and mouse information e.g. UK Biobank and Mousephenotype.org to obtain information which will reduce the number of animals we need to use.

Using our experience and that of the animal technicians, we will employ efficient breeding strategies; for example, mouse lines will only be maintained whilst there is a justified use for their continued breeding. Any line with no predicted usage will be cryopreserved. Prior

to establishment of a colony of GA mice under this licence, any available breeding data will be sought. Well-established breeding calculations will be used to predict output.

Breeding numbers are calculated taking into account average litter size for that stock as well as known neonate mortality. Breeds are set up in a controlled, time-restricted manner, to ensure that all mice born are the correct age for the study.

Where breeding information is not known (i.e. for new lines) a small pilot breed will be carried out first to assess viability. These mice, if viable, can be used for the first cohort of the study, and remaining larger breeds will be set up taking into account information gathered from the pilot breed.

Where possible, we will maximise the data obtained from each cohort of mice. For example, when mice are sacrificed blood, urine and any relevant tissues will be extracted from the same animals. Where relevant, this may include tissues from other organs such as heart, lung, liver which we will share with our existing collaborators.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The severity of the breeding stock will be held to a minimum by the following strategies: Information gathering- Information on any expected adverse effects will be gathered prior to establishment of a line. Welfare assessment - Systems of comprehensive welfare assessment are in place. These will be used to define the least severe humane endpoints for every GA line. Least severe genotype - Where possible the lines will be maintained as the least severe genotype possible (e.g. heterozygotes will be used for stock maintenance where the homozygous state has undesirable adverse effects).

Sampling techniques - The least severe technique will be used to obtain a biopsy for genotyping i.e. from the ear pinnae.

Phenotyping tests - Kidney function analysis will be assessed in metabolic cages. Mice will also be monitored for welfare and imaging techniques will be employed to check their organs.

Where substances are administered to mice e.g. nephrotoxic agents, we will use the minimum amount of agent, by the most appropriate route (typically intra-peritoneal administration) which has previously been shown to cause the desired effect.

Why can't you use animals that are less sentient?

The mouse is the lowest mammalian species in which the full range of genetic and physiological manipulations necessary for the investigation of kidney development and disease can be achieved. It is critical to perform these studies in mammals since there are significant differences between the metabolic systems of frogs and fish to that of humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Increased daily monitoring of animals will take place where substances have been administered, this will include monitoring of body weight allowing us to define more humane endpoints. Pain management will be provided on the advice of the Veterinary Surgeon for any procedure or situation that requires it. Where agents are administered, this will be done as per published protocols, using known drugs. Training is continual, making use of new developments and technologies as they become available.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The establishment where work under this licence will be undertaken is AAALAC accredited. We will follow published ARRIVE guidelines and as well as information issued by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), and the RSPCA. Routes and volumes for administration of substances will be taken from Laboratory Animal Science Association good practice guidelines: administration of substances 1998 (http://www.procedureswithcare.org.uk/lasa_administration.pdf).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The scientific literature and conferences are sources of information on new developments and technologies, as are workshops run at our university on the Replacement, Refinement and Reduction of animal use that I will attend. The establishment where this licence is held often develops and introduces 3Rs advances. Our previous work has developed new tools for research into kidney development and disease using cell lines and urine patient-derived cells that replace animal use by generating material from human urine.

9. Pathophysiology of Rhomboid-Like Proteins in Mammals

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Mouse, Rhomboid, Inflammation, Growth factor, Signalling

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our ultimate goal is to discover whether the rhomboid-like proteins are potentially valuable medical targets. To do this, we will investigate the physiological roles, and possible disease significance, of proteins of the rhomboid-like superfamily.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Research has shown that proteins of the rhomboid-like family are unusual proteases – enzymes that cut other proteins – which specifically target membrane proteins. More recently, rhomboid proteases have been discovered to be members of a much broader

family of proteins whose fundamental role is to interact with other proteins in cellular membranes and to control signalling between cells.

Understanding the signalling between cells and what goes wrong in diseases is a major goal of modern biomedical science. It has also become clear that rhomboid-like proteins participate in many important processes of potential medical relevance, including in cancer, inflammation, pathologies associated with or exacerbated by ageing, diabetes, neurological disease. Much of this prior work has focused on invertebrate model systems and cell culture. Little is yet known about their function in mammals and, despite knowledge of how rhomboid-like proteins work at a molecular level, little is known about how they contribute to health and disease. Very recent work with mice, however, has supported the idea that members of this family are medically relevant, specifically in the field of immune system function, inflammation and cancer. For example, the iRhom proteins have been specifically linked to inflammatory processes including rheumatoid arthritis and heart repair after myocardial infarction. We need to understand whether these proteins are valuable therapeutic targets and, if so, in what specific disease contexts.

What outputs do you think you will see at the end of this project?

This work will illuminate the physiological significance in mammals of novel and fundamental cell biological processes. Such discoveries will be published in open access scientific literature so that it is available to anyone for free. These advances will be used by the wider scientific community to extend the limits of our understanding of biological systems that have ultimate medical relevance. Areas of medicine in which current evidence implicates the Rhomboid-like family include cancer, inflammation, metabolic disease, bacterial infection and parasite invasion.

Who or what will benefit from these outputs, and how?

The immediate beneficiaries will be the scientific research community. In the short term, this will specifically be the worldwide community of scientists working on related areas of rhomboid-like proteins and the regulation of membrane proteins. In the longer term, our hope is that the conceptual discoveries that our work will lead to will benefit biomedical scientists working on a wider range of topics including how cells signal to each other, how protein quality control is regulated and how these cellular processes affect the whole animal. Data will also be very useful for other researchers studying a host of processes related to human health and disease (e.g. inflammation, cancer, neurodegenerative diseases) and we will publish it in appropriate journals to ensure that all relevant researchers can benefit from this work.

Longer term beneficiaries will be the public, through expected improvement in health care. Specific areas that are of current interest include cancers, especially of the lung and oesophagus; neurodegenerative diseases like Alzheimer's Disease; and metabolic diseases such as diabetes. But the fundamental significance of the biology we study means that many other diseases may also become relevant as we learn more. These

ambitions are not imminent but our work seeks to build the foundations for new therapeutic and/or preventative strategies. To realise such potential, we will work in collaboration with pharmaceutical or biotechnology companies as appropriate.

How will you look to maximise the outputs of this work?

We frequently find it is more efficient to collaborate with other expert groups to maximise our ability to exploit our discoveries. The nature of our research leads us into a wide range of physiological and disease contexts where our own experience is limited. Well targeted collaborations accelerate progress.

We aim to publish in open access, free to all, scientific literature all our research that is rigorous and reproducible, regardless of whether the results are positive or negative. Disseminating knowledge about unsuccessful approaches can be as valuable as more obvious breakthroughs. We now publish all our work on preprint servers so that our results can be distributed prior to the slower process of peer reviewed publication in leading international journals, where all the work will eventually end up. Our work will also be communicated through conference presentations by me and members of my group.

We have frequently discussed with the commercial sector the potential exploitability of our research. These conversations are ongoing, continuously looking for opportunities that can be pursued by those routes.

Species and numbers of animals expected to be used

- Mice: Mice, up to 10,000 over 5 years.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Because our starting point is understanding fundamental cellular mechanisms, our work always starts with biochemical and cell culture experiments that do not involve animals. As we begin to reveal those basic mechanisms, we then need to apply that emerging understanding to animal models to ask the question of the overall biological significance of what we are studying. The physiological role of proteins can vary substantially between species, explaining this need to validate all the previous work in a system much closer to human biology. Specifically, mice are the organism of choice because they are mammals, and thus quite close to humans in evolutionary terms. They are also the mammal with the most sophisticated genetic technology available to allow us to perform our experiments with maximum efficiency. Without these experiments, our work cannot be used to infer the real significance of the cellular mechanisms in whole animals, reducing its potential benefit.

A few specific examples of rhomboid-like proteins have been implicated in disease processes including their role in the invasion of host cells by apicomplexan parasites, which include the malaria parasite Plasmodium; their now well established central position in promoting and regulating inflammatory signalling, which in turn has relevance to diseases as diverse as rheumatoid arthritis, cancer, metabolic disease, and neurodegeneration; and their role in regulating T cell immune homeostasis, growth factor release in cancer, and mechanisms of antibiotic resistance in some bacteria.

The clear preliminary evidence, outlined above, of the potential medical significance of members of the rhomboid-like superfamily, provides the incentive and justification to learn about their physiological role in mice and, by extension, humans.

When we do use mice, they can be at any stage of life. Most of our experiments are performed on adult mice. But we also occasionally use pre-weaning animals if we see evidence that our genes of interest affect those early stages. We also allow some mice to live to old age, to investigate potential roles in aging of the genes we study. For example, as humans age, systemic inflammation is associated with neurodegeneration, so the link between rhomboid-like proteins, inflammation and neurodegeneration may have relevance to human age-related disease.

Typically, what will be done to an animal used in your project?

Most of the mice we use are genetically modified to alter genes that we are interested in. The mice are obtained by breeding, non-surgical embryo transfer and, in some rare cases, surgical embryo transfer. To infer the roles of the genes we have modified, we then typically observe them to look for changes to their development, physiology or health. Beyond breeding the genetically altered animals, the most common experiment is to humanely kill the animal and then dissect and study their organs post- mortem. In some cases, to assess the potential role of the genes of interest in ageing or metabolism-associated pathologies, we will age the mice and use models of diabetes. The duration of these experiments is kept to the minimum that allows us to collect the necessary data.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the animals used under this licence will experience no detectable clinical harm or suffering until they are humanely killed before they are 15 months old. They will simply be bred with an alteration in a gene that does not detectably affect them.

For some of our experiments, we will age mice up to 2 years. Ageing mice may develop conditions such as dermatitis, cataracts, dental diseases or tumours. Animals that show signs of ill health associated with any of these or other conditions will be killed humanely.

One of our protocols can lead to the onset of diabetes. In this protocol, we will keep mice up to 1 year on a high fat diet. Blood glucose level will be measured in these mice to monitor the onset of diabetes.

Some genetically modified strains that we currently maintain can produce moderate clinical signs. Although some homozygous genetically modified pups die within a few hours of birth, those that survive more than a day are indistinguishable from their heterozygous and wild-type littermates until about 10 days after birth. Around day 10, these surviving homozygotes become recognisably smaller than normal, but appear otherwise healthy and show no other signs of harmful effect. Between days 14 and 21 (exceptionally later), they develop signs of failure to thrive and rapidly decline; if left, they die within 2-3 days after that stage. The signs that we have detected in this final phase include subdued behaviour, prostration and weight loss. Pups of these strains are therefore monitored and weighed daily from day 5. All these animals gain weight, although the homozygous genetically modified mutants do so at a slower rate. Eventually, the homozygous mutant animals start to lose weight. When the weight of an animal drops by 15% from its peak, it is humanely culled. This should prevent any animal from showing signs of suffering but, in addition, any mouse will also be culled at the first indication of prostration or subdued behaviour.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: 80% Sub-threshold, 5% Mild, 15% Moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice are well established as the best model system for identifying the function of specific genes in mammals. Usually this is done by removing or knocking out the relevant target gene, or by expressing it in abnormal places or levels. The consequent effects on the mice or on cells derived from them are observed and they give strong clues about the function of the gene/protein of interest. In fact, we do all of our initial research in cell culture and using the fruit fly Drosophila. This allows us to obtain most of our results without using mice, thereby minimising potential suffering. However, where we need to extend our results into mammals, to investigate their relevance to human and animal health, we need

to use mice. For example, we have evidence that the iRhom1 and iRhom2 genes regulate inflammation; that the TMEM115 gene is involved with metabolic control; and that the RHBDL2 gene regulates immunity. We need to use mice to explore whether these functions are physiologically and pathologically significant. These questions cannot be addressed in cell models or Drosophila.

Which non-animal alternatives did you consider for use in this project?

- We do as many of our experiments as possible in vitro, in tissue culture cells. For example, we always start by creating new mutant cells that can be used to investigate much of the fundamental biology without using mice. This is usually the best approach to analysing molecular and cellular mechanisms. We limit our use of animals to asking questions about whole organism consequences of our experimental manipulations.

- We use in silico databases (such as Protein Atlas, BioGPS, UniGene, etc) to check the patterns of expression of the genes of interest, avoiding the need to take samples from mice.

- We systematically monitor information from mouse knockout projects (for example the International Mouse Phenotyping Consortium) to ensure that we do not remake GM strains that already exist and to capitalise on their existing phenotyping data. Where possible, we can import relevant GM lines, avoiding the need for us to remake them.

Why were they not suitable?

Ultimately, the information we acquire from biochemistry, cell culture and computer-driven approaches needs to be validated and understood in the context of whole animals, which are much more complex systems than these reductionist approaches can explore. At that stage in our work, we need to be able to ask the question about whether what we have discovered does apply to whole animals. We typically address this question first in the fruit fly Drosophila, but need ultimately to extend the logic into mammals. Only where appropriate GM strains do not exist in publicly accessible databases, do we need to make them ourselves.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We carefully design all our experiments to ensure that they are as efficient as possible, so that we can produce the maximum amount of statistically rigorous data from the minimum

number of animals. For example, in the case of majority of our mouse work, breeding GA animals, this means that we first calculate how many mice we will need to analyse a phenotype. Then, based on our prior experience, we only breed that number, producing as few mice as possible. In the smaller number of cases where we need to do an experiment that involves treating mice with substances the same principle applies:

we determine in advance, using a pilot experiment, what is the smallest number needed to produce statistically robust data, and we use only that number in our experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our focus on using systems other than mice for all the early stages of our work, combined with planning to breed only the number of mice we specifically need, make us confident that we are using the minimum number of mice possible for our work.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As described above, our mouse work is a very limited proportion of our overall experimental approach. As well as efficient breeding, pilot studies, sharing data and tissues with collaborators, our whole approach is to use animals only when there is no alternative.

We also consult mouse phenotype databases to ensure that we do not duplicate work that is already in the public domain; this information also helps us to give priority to genes with phenotypes expected to be of interest.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Wherever available, we use genetic models of disease that are the most precise and efficient way of mimicking in mice the relevant human disease. We take great care that all procedures done on mice are performed by state of the art methods and only by people who have appropriate training. We use a range of scientific communication methods (work published in journals, conference presentations, informal networks, online resources) to learn about best practice so that we can ensure that our methods are as refined as

possible. We hold regular team meetings to ensure that this information is spread across our group, and with collaborators. We also attend regular meetings among the wider scientific community to stay current with best practice.

To assess possible metabolic defects, where there is mechanistic evidence to suggest a role of a specific rhomboid-like protein (eg in the case of TMEM115) we will maintain mice on a high fat diet. This is a standard method to induce obesity and type II diabetes in mice. This is a non-invasive model that will allow us to address if specific proteins of the rhomboid-like superfamily affect onset of obesity and diabetes.

To assess any age associated phenotype, we will maintain mice up to 2 years. This is just natural ageing and is therefore the most refined technique to allow the observation of any late onset phenotypes. As mice become older, after 12 months, we pay extra attention to their health and observe them carefully for any clinical signs.

Why can’t you use animals that are less sentient?

Our general approach is to perform most preliminary organism studies in simple invertebrate model organisms including the fruit fly Drosophila, or even in yeast. Not only do these systems allow much more rapid mechanistic progress than using mammalian models, but this approach also reduces substantially the need to use mammals for early phases of our experiments. A successful outcome of the Drosophila work allows us to have a clearer view about the physiological role of a gene. This approach is based on the highly conserved nature of rhomboid-like genes. Although the overall physiological role of rhomboid-like proteins in mammals cannot be inferred from their function in invertebrates, all current evidence supports the conclusion that their basic cellular mechanisms are conserved. Ultimately, however, it is essential to extend our invertebrate studies into mammalian model systems, partly to validate the models, partly because however good a model is, mammalian biology is almost always more complex, and partly because some of the genes we study (eg RHBDD2) are only present in mammals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To minimise the welfare cost of procedures, we will monitor carefully animals for any sign of adverse effect. If an animal exhibits any adverse effect, we will take action according to procedure detailed on each specific protocol which include increased monitoring and attempt to ameliorate these initial signs. For example, in some specific cases we will maintain mice on high fat diet for up to a year. During this procedure, blood glucose level will be measured to detect the onset of diabetes. Also, during ageing, animals will be carefully monitored for age-associated conditions such as the presence of palpable masses, skin abnormalities, or dental overgrowth. After surgical embryo transfer under general anaesthesia, we will monitor how the animal recovers from the anaesthetic. If the animal exhibits any sign of pain, peri-operative analgesia will be administered and continued after surgery for as long as required to alleviate pain.

For genetically modified strains that can exhibit moderate clinical signs, such as TMEM115, we set up a more refined breeding strategy. For these strains, it is only the homozygous genetically modified animals that exhibit moderate clinical signs; the heterozygotes are indistinguishable from wild-type littermates. We therefore maintain the colony with heterozygous animals by crossing heterozygotes with wild-type. Only when we need homozygous animals for an experiment do we cross heterozygotes with heterozygotes to produce homozygous pups. Some of these homozygous genetically modified pups die a few hours after birth, and those that survive longer show signs of harmful phenotypes within a few weeks after birth. To reduce suffering of these pups, we perform most of our experiments just after birth. Only when it is experimentally required, do we maintain homozygous animals for a few weeks. In this case, pups are weighed daily and closely monitored for any sign of harmful phenotype.

When the weight of an animal drops by 15% from its peak, it is humanely culled. This should prevent any animal from showing signs of suffering, but any mouse is also culled at the first indication of prostration or subdued behaviour.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure that experiments are conducted in the most refined way, we rely on best practice guidance provided by the Named Information Officer and available on the following websites:

www.nc3rs.org.uk https://norecopa.no https://www.lasa.co.uk

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly monitor information from the NC3Rs (www.nc3rs.org.uk); there are active email circulation lists operated by the university's named information officer; we attend termly animal welfare meetings in which developments in 3Rs are regular agenda items, often led by expert speakers; and we are greatly supported by the local network of Named Animal Care Welfare Officers, vets and animal technicians, all of whom have great experience in animal welfare and experimentation, as well as having their own networks.

10. Developing a Treatment for Osteogenesis Imperfecta

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

collagen, osteoblast, therapy, b**ones, stem cells**

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to test the efficacy of innovative treatments to counteract bone fragility.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In human, osteogenesis imperfecta (OI) is a rare genetic disease characterised by bone fragility, short stature and skeletal deformities in response to a genetic mutation in the genes encoding type I alpha 1 or alpha 2 pro-collagen. This is a severe and debilitating skeletal disease that starts to manifest in the womb. There is currently no cure for this pathology. This project tests the potential of new therapeutics to improve skeletal health, with the view to translate these findings to the clinic.

What outputs do you think you will see at the end of this project?

Our outputs will include:

- publication of results in peer-reviewed journals and presentation of the project at national and international conferences in relation to the mechanisms of OI disease progression and the development of innovative therapeutics

- press releases and communication of findings with the public via social networks

- communication of findings with the OI community via social platforms and conferences

Who or what will benefit from these outputs, and how?

- in the short term, this work will benefit the researchers working on this project (career development,) and other scientists working on developing bone anabolic agents.

- in the long term, this project will benefit clinicians because we will develop and test new therapeutics.

- beneficiaries also include our ongoing clinical trial (BOOSTB4), which is currently testing the safety and efficacy of stem cells injection.

- ultimately, the long-term benefit will be the improvement of the quality of life of children with OI and that of their families.

- reduction of care costs for the healthcare system

How will you look to maximise the outputs of this work?

To maximise the outputs of this work, we are closely working with our collaborators who are also using OI mice colonies to study cellular stress in response to mutations in genes encoding type I collagen. As we are collaborating with this group for another project using the same mice, we will benefit from their advice on this animal model.

New knowledge will be distributed internally (work in progress and seminars) as well as externally (publications, conferences, press).

Species and numbers of animals expected to be used

- Mice: 3500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using this mouse model of brittle bone disease because it is the most widely used and a good model for severe osteogenesis imperfecta (OI) in humans.

We currently have an ongoing clinical trial (BOOSTB4) where babies are injected with stem cells in utero, at birth and repetitively thereafter as we have documented that the effects of mesenchymal stem cell (MSCs) transplantation do not last long. This is due to the dynamic biology of the skeletal system, where old bone is constantly resorbed and new bone deposited.

The cells currently used for the clinical trial are isolated from the human liver of terminated fetuses, expanded in the laboratory to obtain sufficient cells, and injected in OI patients.

However, this may raise ethical issues for some patients as mesenchymal stem cells are isolated from terminated donors. In addition, when we expand the mesenchymal stem cells in the laboratory, those age quickly and therefore we need to isolate and characterise more cells from new donors on a regular basis.

We are now testing the possibility to replace the use of human fetal MSCs isolated from termination of pregnancy by another type of MSCs. Specific cells (epithelial cells) found in the urine of the patients are being rejuvenated (induced pluripotent stem cells), subsequently edited to remove the genetic cause of the disease (personalised medicine), before being differentiated into induced mesenchymal stem cells (iMSCs).

Therefore, we are using this mouse model of the disease to determine the efficacy of iMSC injection and other treatments, such as transplantation of the vesicles released by iMSCs (to develop cell-free therapeutics) in order to translate this information to humans and develop the next generation of cell therapy for the treatment of bone fragility.

Typically, what will be done to an animal used in your project?

Typically, pregnant mothers will be left with their litter (from heterozygous x heterozygous breeding or from homozygous x homozygous breeding) until weaning. The pups will be injected intraperitoneally at birth (0-5 day of age) with a sterile saline (PBS) solution containing either human or mouse cells, exosomes, or only sterile PBS (not containing cells nor exosomes), or not injected al all. The pups will then culled at 8 weeks of age.

What are the expected impacts and/or adverse effects for the animals during your project?

There is no expected adverse effect of the treatment (intraperitoneal injection of cells, saline solution or exosomes) for the animals. Our previous work showed that stem cell injection decreases long bone fracture rate by 2/3.

The presence of the naturally occurring mutation is associated with fractures, small size and skeletal deformities in homozygous mice only. It is possible that homozygous mice may exhibit other pathologies such as poor mobility, limping, swollen limbs/joints, signs of pain and discomfort.

Heterozygous mice do not show any phenotype because the mutation responsible for bone fragility is recessive.

Our previous work using the same model and same endpoints has established the potential of human fetal mesenchymal stem cells (MSCs) isolated from the liver of terminated fetuses to counteract bone fragility in this model which best mimics severe human brittle bone disease. The current work aims to test the efficacy of a different type of MSCs called iMSCs, i.e. instead of being isolated from terminated donors, iMSCs are differentiated from induced pluripotent stem cells that have been derived from the epithelial cells found in the urine of the patients following a genetic correction of the disease-causative mutation. We are also testing the efficacy of only transplanting the vesicles released by MSCs to develop personalised cell-free therapeutics.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

When we will cross heterozygous males with heterozygous females. The mutation is recessive. Therefore we will obtain 25% homozygous pups, 25% wild type pups and 50% heterozygous pups. Only homozygous mice present a harmful phenotype of bone fragility.

The heterozygous mice are not phenotypically different from wild type mice and do not present any harmful phenotype (they do not have skeletal deformities and have no fractures).

Based on our previous work (PPL 70/6852), we expect the breeding and maintenance of heterozygous mice to be mild ; breeding and maintenance of homozygous mice to be moderate and the experimental mice to be moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are using an animal model of a human pathology to be able to analyse the impact of the mutation responsible for the disease on the skeleton in various bones, and to determine the efficacy of cell and cell-free therapy on improving the shape and strength of the whole skeleton at the macro, micro and nano level. These analysis will be performed post-mortem.

Which non-animal alternatives did you consider for use in this project?

We are currently developing a human bone-on-chip model of the disease to study the impact of the treatment at the cellular level.

Why were they not suitable?

The in vitro disease model has several limitations. For example it is not possible to study fracture incidence, skeletal morphology and long bone structure and mechanical properties at a macro level.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

A statistician has been involved in the preparation of the proposal from. We will use a fully randomized design to reduce the biological variability. We used the power analysis software G*power version 3.1 to determine the number of mice/group, 2 tails, a power of 0.95, and a significance level <0.05, with n1=n2. We determined sample size using the data we obtained in our most recent study where we showed that stem cells improve OI bone properties and quality following a single injection into oim neonates. We will analyse the bones of transplanted mice in a similar manner and therefore will have the same endpoints. Trab number: 1.3±0.19 (mean±STD) vs. 1.7±0.2; ES=2.05; n=8; Bv/Tv: 3.9±0.7vs. 5.2±0.7; ES=1.85; n=9; Bone min density: 0.03±0.01 vs. 0.06±0.01; ES=3; n=5; Total porosity: 7.2±0.7 vs. 5.4±0.5; ES=2.96; n=5; In consequence, we need about 10 mice per sex analysed per group (so 20 mice per group) to attain sufficient statistical power for microCT analysis. We counted 30 mice transplanted per group, in order to take

into consideration a 25% possibility that the bones might be broken and hence unsuitable for analysis, and 25% possibility that other cell types might have a smaller effect than the cells we studied before.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use homozygous mice to study the impact of the treatment with cells or exosomes on bone strength. We will analyse the results as we go along to ensure group size are minimal (using power calculation).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All the bones from experimental animals will be used for analysis. We (and our collaborators) do not have any archived tissues for that purpose.

We do not have any tissues archived to be shared with collaborators and vice versa.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The osteogenesis imperfecta mouse (oim, B6C3Fea/a-Col1a2oim/+) has been used as a model for human osteogenesis imperfecta (brittle bone disease). It was the first animal model of human osteogenesis imperfecta and has been extensively studied.

Why can’t you use animals that are less sentient?

Because this model presents the same phenotypical characteristics as the human condition and enable the efficacy of treatment to be studied at the macro level.

We cannot use strains of OI Zebrafish as those lack the impact of weight bearing on the skeletal, and therefore do not represent the best model for the human pathology.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice will be handled by empathetic capture and handling (e.g. tunnel capture or cupped hands) rather than by scruffing or by the tail, to minimise fractures. To reduce the risk of bone fractures in homozygous mice, easily accessible food and water (e.g. soft/wet/powdered food and TransGel/HydroGel at floor-level) will also be provided. Oim homozygotes are smaller than wild-type or heterozygous mice but animals showing visual signs of weight loss will be provided with appetising food supplements in the first instance such as peanut butter or condensed milk. Animals will be provided with sufficient non-tangling nesting material, refuges to permit natural behaviours, defuse aggression and alleviate anxiety as well as floor-level enrichment such as tunnels. However, to reduce the incidence of fractures, balconies will be removed from the cages to reduce the impact on bones after jumping. We will use special labels to seek attention to the homozygous mice.

We will use the welfare sheet previously developed by ourselves and our collaborators in consultation with the NACWO and NVS, which details husbandry considerations and refinements for the strain. The welfare sheet will include the minimally invasive daily checks that should be carried out as well as the humane endpoints. The sheet will specify whether and how the mice should be handled to avoid fractures. The welfare sheets will then be further refined as the study progresses.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

National Centre for the 3 R's: https://www.nc3rs.org.uk/

LASA guidelines: https://www.lasa.co.uk/PDF/AWERB_Guiding_Principles_2015_final.pdf
Understanding Animal Research: https://www.understandinganimalresearch.org.uk/

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

we will regularly check the Home Office website https://www.gov.uk/guidance/research-and-testing- using-animals and the NC3Rs website https://www.nc3rs.org.uk/. We will also remain in close contact with our collaborators.

11. Immune Cell Function in Cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, metastasis, immunology, genetically engineered models

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand the role of the immune system during cancer progression, cancer spread (metastasis) and therapy.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This work is important to gain fundamental insights into immune cell function during cancer progression, metastasis and therapy. These results may be useful to develop immunotherapies in the future.

What outputs do you think you will see at the end of this project?

Data generated from this project will include new information on the role of immune cells in cancer progression and spread (metastasis), which will result in publications and datasets that will be made publicly available.

Who or what will benefit from these outputs, and how?

These findings will benefit researchers studying immunology and cancer biology, as this will provide a greater understanding of the connection between cancer cells, immune cells and other components of the tumour microenvironment and of the mechanisms that drive cancer progression and metastatic disease. The impact of these benefits are likely to be long-term, as it will enable researchers to generate more informed hypotheses and will improve experimental design in accordance with the 3 R's (replace, reduce and refine).

How will you look to maximise the outputs of this work?

We expect the output of this work to lead to the establishment of collaborations with other research groups working in similar fields. We will look to disseminate any new knowledge by manuscript publication and the presentation of novel data at conferences to seek out potential collaborators. We will also promote new developments through social media networks as a way of informally communicating with scientific colleagues, but also sharing our findings to the wider public.

Species and numbers of animals expected to be used

- Mice: 45000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Genetically engineered mouse models of cancer are the most representative models of human cancer available, since they progress through the same stages as in humans and metastasize to the same sites. Our choice of mouse models is based on the same mutations that occur in human cancer. In these models, tumours arise in adult mice.

Typically, what will be done to an animal used in your project?

Mice with genetic alterations will be bred and used in experiments. The majority of mice in the project will not suffer any adverse effects because they will not express all the right gene mutations to develop cancer. With regard to the mice carrying the right gene mutations, most will develop cancer as adults and be humanely killed without additional procedures when they show symptoms of cancer. Cancer will be induced in some mice using an injectable agent, typically on one occasion only. In a small number of cases, in order to study particular key aspects of human disease, including the process of metastasis, surgical techniques may be used to implant tumour cells grown in the laboratory into organs affected by metastatic disease, such as the liver or the colon.

Some mice will be used in preclinical trials, in which they will be administered with treatments designed to potentially benefit human patients. These treatments can include drugs, which can be administered orally or by injection, or can include radiotherapy using a specially designed small animal radiotherapy machine. In a small number of cases, tumour growth and response to treatment may also be monitored using advanced imaging techniques which are commonly used for human patients in the clinic, such as MRI, ultrasound or CT scanning. This will require mice to be anaesthetised for short periods of time while images are collected.

All experimental animals will be humanely killed and tissue specimens collected for analysis to maximise the data available from every study.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of mice in this project will have a subthreshold severity, meaning that they will show no harm, and will undergo no procedures other than earmarking for identification purposes. About a quarter of animals (25%) will have a predisposition to develop cancer, of which a proportion will exhibit no more than mild adverse effects, as they will be sampled at timepoints prior to manifestation of clinical symptoms of ill-health. The remaining animals will be expected to develop moderate adverse effects associated with tumour development. With respect to procedural burden (e.g. imaging, therapeutic intervention, metabolic labelling), some animals will experience one or more procedures, which will never exceed a moderate severity.

Under this project, cancer-related genetic changes will be targeted to a tissue of interest, such as the intestine, pancreas, liver or mammary gland, which will result in a specific predisposition to cancer development in that tissue. These animals will then be continually monitored for clinical signs which may indicate tumour growth. These signs can include anaemia, weight loss, swelling of the abdomen and development of palpable or visible tumours. Highly trained staff will monitor for these symptoms, and they are observed to interfere with normal behaviour, reach the limits allowed in the guidelines or have any consequences that are outwith the guidelines, mice will be humanely culled and tissues harvested for analysis. Typically mice might exhibit mild clinical signs, which do not impact

normal behaviour for around 1-2 weeks, and moderate clinical signs for 24-48 hours before they are humanely killed. Animals which undergo surgical implantation of tumour cells under anaesthetic, may experience transient discomfort (~48hrs), which will be alleviated through administration of pain relief.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Tumour-bearing mice typically experience moderate severity, while tumour-free mice experience sub- threshold severity. Across all cohorts, we expect that 50% of animals will experience sub-threshold discomfort with 50% of animals experiencing mild or moderate discomfort.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Genetically engineered mouse models (GEMMs) of cancer are the most representative models of human cancer and can be easily manipulated to enable tumour development. These models are driven by a combination of activated oncogenes and mutated tumour suppressor genes that occur in the human cancer, and these display similar tumour progression, metastatic dissemination and clinical manifestations found in human disease. Additionally, mice with a fully competent immune system can be used to study the dynamics of tumour cell/immune cell interactions during tumour development.

Combining GEMMs with the loss of specific immune system components (such as genetic depletion of individual cell types) then enables the impact on tumour growth and metastatic dissemination to be assessed in the context of immune dysregulation.

Which non-animal alternatives did you consider for use in this project?

Where appropriate, non-animal alternatives such as cell culture will be used to address our objectives, in accordance with replacing and reducing animal experimentation. Often this is considered when investigating a mechanistic pathway following a significant finding from animal modelling.

Why were they not suitable?

It is not feasible to study the complex interactions between cancer cells and immune cells or the crosstalk between different immune cells in humans and animal models. To accurately model the tumour microenvironment and provide more faithful models of tumour progression and metastasis, a whole immunocompetent organism is required. Mice contain specialized organs that lower organisms do not possess, specifically an intact immune system which enables us to study the co-evolution of immune responses with tumour development.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used were based on previous usage, breeding pairs required, output of breeding pairs with correct genotype for each model, experience with each model and power calculations for specific experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We make use of multiple tissues from every mouse so that less animals are needed. We also routinely perform power analysis to determine the cohort size needed to achieve significant results, this is often informed by pre-existing data gathered from pilot experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breedings are carefully designed to establish efficient breeding pairs, which in turn reduces the number of mice needed to generate tumour-bearing cohorts. Breeding pairs are also closely monitored to avoid animals reaching ages where adverse effects are evident, and we also monitor breeding efficiency to prevent excessive breeding pair numbers.

Often we implement pilot studies, and try to use cell culture models where appropriate, alongside power analyses to predict cohort sizes that would return significant results, which reduces the numbers of mice potentially used throughout this project.

Finally, when establishing a project we often bank various tissues/organs for future use, which prevents the unnecessary generation of additional mice. Furthermore, the

collaborative nature of our research encourages the sharing of tissue if needed for pilot studies, which reduces the number of mice used across multiple projects.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetically engineered mouse models of cancer as well as tumour transplantation models because they contain an intact immune system. Whilst spontaneous tumour development is a central part of this project, appropriate steps will be taken to ensure the wellbeing of animals and to minimise the potential harms may have upon normal animal behaviour. We do not seek to keep animals that are suffering significant adverse effects and plans as far as possible to avoid animals reaching the stage where adverse effects are present. Mice are checked daily by animal staff as part of welfare checks, and up to three times weekly by researchers to monitor for any unexpected or expected phenotypes during tumour development. We have extensive in-house experience with the models detailed in this project, and have well-defined limits of humane endpoints, which are applied to prevent excessive suffering.

Why can’t you use animals that are less sentient?

We will use adult mice with a fully competent immune system to mimic cancer in humans. A fully competent immune system is crucial, as tumours develop within an inflammatory fibrotic matrix that influences tumour progression, metabolism, invasive potential and metastatic dissemination. This is best modelled in mice with competent immune systems that develop tumour spontaneously and which mimic human disease through genetic mutations, disease progression and clinical manifestations.

Terminally anaesthetised animals are not appropriate, as we aim to study the progression of tumour over months, where the co-evolution of immune responses and tumour growth will be studied.

Additionally, this co-evolution cannot be modelled in less sentient animals, or through experiments in the lab, as they do not recapitulate an immune competent setting.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Currently, we use frequent monitoring, post-operative care, pain management and advanced training of staff to minimise harm to animals. We have adopted non-aversive handling methods and avoid single housed animals to reduce stress and encourage social behaviour. We will continue to review our practices over the course of the project to ensure that we use the most up-to-date refinement methods available.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will adhere to ARRIVE guidelines and the NC3Rs strategy documentation to ensure compliance.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Me and my team will regularly visit the NC3Rs website to remain up-to-date with 3R resources, the publication of new methodologies and also to ensure adherence to ARRIVE guidelines. We will also attend conferences where we can discuss the implementation of 3R policies with other members of the scientific community.

12. Breeding Genetically Altered Mice

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Breeding, Maintenance, Reproducibility, Supply, GA mice

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To breed and maintain genetically altered mouse models relevant to the study of normal and abnormal biological and pathological processes in mammals. To carry out breeding as a service to scientific groups throughout the UK and occasionally abroad.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Undertaking this work as part of a standardised breeding programme is essential to increase reproducibility, maintain the genetic integrity of mice used in genetic research and offers the opportunity to reducenumbers for specific projects.

1) Breeding cohorts under one breeding licence allows us to control the breeding strategies and ensure background strains are consistent, genetic backgrounds reproducible and describable for publication. We can also ensure that controls and experimental mice are bred using the most appropriate breeding strategy to minimise experimental variables and cohorts are produced in appropriate timeframes.

2) Breeding of newly generated GA lines will be done on this licence before they are sent to researchers. This allows us to increase stock numbers and archive the lines, whilst gathering important welfare information and carrying out any additional Quality Control (QC) measures to ensure newly modified alleles are fully characterised.

3) Quality controlling inbred strains of mice ensures that subsequent experimental cohorts bred using these strains have a known, reproducible genetic background. Conclusions about the phenotype caused by the introduced genetic alteration are easier to interpret and more robust and reproducible using inbred strains rather than non-standardised or uncontrolled backgrounds.

4) Central holding of GA stocks means that several researchers can be supplied from the same colony without the need to keep duplicate colonies which would use more mice.

What outputs do you think you will see at the end of this project?

1. Cohorts of mice will be maintained and bred for specific projects and transferred to other project licences either at the primary establishment, or at other establishments. Cohorts of mice will also be bred for tissue preparation and collection at the establishment. These cohorts will be used for many types of research in many different areas. Often the outcomes of these projects will lead to a greater understanding of biological mechanisms and underlying disease. In turn this can lead to better diagnosis of human conditions, and the potential to use the mouse as a model to test therapeutics. It is difficult to predict the numbers accurately as these will be dictated by scientific justification of the individual projects, however we are likely to breed around 30-50 of these cohorts per year. These mice will be bred under strictly controlled conditions, for example producing age matched cohorts with relevant controls on an appropriate background. By using our extensive knowledge of breeding GA lines and keeping ourselves current with recent developments in the field, we will strive to produce cohorts of mice that are high quality. The alternative would be to ship breeding pairs which would require multiple breeding steps at another establishment. Many establishments do not have access to facilities available at the establishment, such as supply of quality controlled inbred wild types, quick and efficient genotyping, an extensive database in which to track all breeding parameters and knowledge of breeding a large variety of GA strains, both in terms of mouse welfare and complexity of alleles. This could result in mice being bred on mixed or inappropriate background strains, cohorts being produced over unsuitable time frames using inefficient breeding schemes, and suboptimal production of control mice, leading to an increase in the variability of results as well as reduced reproducibility.

2. A number of mouse lines for a peer reviewed initiative to refine and deliver better mouse models for biomedical research will be bred on this licence. These lines will be established on this PPL and bred for dissemination to the biomedical community. It is anticipated that around 30 new genes will be modified per year, with the potential for multiple different alleles being produced for each gene. The mice may then be transferred to different PPLs in order to phenotype and characterise the lines further. All mouse strains from this initiative will be archived and freely available to the scientific community. Carrying out the initial breeding steps on this licence allows full quality control (QC) of the modified alleles, including checking for the off-target effects of genome editing and fully sequencing the target locus to check for any unwanted changes (Mianne. 2017). Carrying out these QC steps is critical to properly characterise the lines and understand what genetic changes have been made. This ensures correct interpretation of how the genetic change impacts the phenotype and ensures lines with unwanted changes are discontinued in a timely manner. Additionally, breeding for another generation before supplying allows welfare data to be collected and passed on to researchers using these strains in the future.

3. Mouse lines for a peer reviewed programme encompassing researchers around the UK will initially be bred on this licence. The programme is a multi-million-pound initiative to bring together clusters of researchers from around the country and partner them with experts in mouse genetics. The aim of the clusters is to accelerate understanding of human disease and improve diagnosis and treatments. The research clusters will have individual PPL's for experimental characterisation in their specific discipline (including cancer, mitochondrial disorders and neurodevelopment), however initial stages of breeding may be carried out on this PPL for practical reasons such as QC and use of shared lines/resources.

4. Wild type mice will be biopsied on this licence in order to perform genetic testing thereby ensuring they are of high quality. Frequent checking highlights any gross contamination and more in-depth sequencing (done less frequently) informs us of the genetic integrity of the inbred strains and helps inform when we should restock in order to reduce genetic drift. If these checks were not carried out, the mice could be bred without the need for a project licence, however, we would be unable to detect any contamination in the background and would have no information of the genetic drift of the colony. The subsequent reduction of genetic integrity could have serious effects on phenotyping results and confound many experiments.

5. GA lines that are in the intermediate stages of genome editing will be bred. With more complex editing using CRISPR/Cas9 becoming possible, there are often occasions when either, a large genetic change is needed that takes multiple steps, or, changes need to be compounded on top of one another. For example, a specific mouse allele may be altered to introduce the human sequence and

then the humanised allele may require the addition of point mutations to mimic a human patient cohort. In these cases, GA mice will need to be bred to supply the genome engineering team for generating further complex strains.

6. On this PPL we will breed standardised GA stocks that are needed by multiple users. This includes common recombinases and reporter lines. Keeping these mice in one colony allows us to reduce numbers, which would inevitably be higher if each group were to keep their own colony, it will also minimise genetic drift.

Who or what will benefit from these outputs, and how?

The expected benefits of this project are production of high-quality GA mice or tissues for research in biomedical sciences.

In the short term this will lead to more reproducible experimental data. Longer term this will have lasting impacts for basic research, as well as better understanding of the benefits and caveats of using mice for models of aspects of human disease and pre-clinical models for drug development. With more appropriate control strategies and standardised cohorts, models will be better understood, allowing them to be used more specifically for drug development and understanding of disease mechanisms.

New lines generated through peer reviewed programmes will be supplied to approximately 16 Higher Education Institutions per year.

How will you look to maximise the outputs of this work?

The knowledge gained through constant re-evaluating of our breeding strategies feeds directly into training courses that we run several times a year on managing mouse colonies. Several members of the establishment breeding team are also members of an NC3R's working group that have written advice on breeding and are available to question through an email helpdesk.

All new lines have welfare information gathered and the archive is annotated with any relevant information so it can be passed on to future researchers.

The Scientific Manager and Director frequently speak at conferences on reproducibility in mouse research, focussing on what we can do about the breeding and environment to produce the best possible research.

Any specific welfare issues of interest to the community will be published, for example, tarsal damage in C57BL/6N mice.

Species and numbers of animals expected to be used

- Mice: 135500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are needed for this project as it is a service licence to supply genetically altered mice to other research programmes. There is no need to age mice on this licence, but we will need mice at all other life stages for breeding.

Mice bred on this licence will be used to supply projects that have been through peer review and/or have a PPL that has been assessed for the necessity to use mice. For GA projects not part of large national programmes, researchers will provide information on peer review and the assessment of alternatives to animal use. For projects where peer review is more difficult to assess (e.g. small biotech companies) requests to use the breeding licence will be assessed by the AWERB.

Typically, what will be done to an animal used in your project?

Mice will be born, they will have an ear clip taken for identification and genotyping and then they will be housed, either for transfer to another licence/project, kept as stock for potential future breeding, or breed with other genetically altered or wild type mice. Any mice not moved on to other projects will be killed. Embryos may also be harvested for tissues after killing.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of mice on this licence will have no adverse effects. In the case where we are breeding experiment cohorts for transfer to another licence the majority of time these mice will be transferred to other projects with authority to use them before adverse effects develop. For lines where adverse effects are unknown, new lines, or crossing certain combinations for the first time, mice will be carefully monitored. It is not the intention to keep mice with adverse effects on this licence, since for breeding it is preferable that all mice are healthy. Mice will only be kept if they are needed for breeding and the adverse effects are unavoidable (e.g. the mice cannot be bred efficiently with a different genotype, the adverse effects cannot be ameliorated by treatment) and within the humane endpoints. In cases where individual lines have known adverse effects that are present before mice can be transferred to the experimental licence or that are present in breeding stock, these will be individually justified in the protocol.

It is possible that some crosses will result in some genotypes being sub-viable or lethal. In these cases mice may not be born in Mendelian ratios, or they may die suddenly soon after birth, before weaning or very occasionally later in life. For lines where there is a

known higher than expected mortality rate the expected rate and details will be justified on a line by line basis in the protocol.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

90% of mice on this licence will have a severity of mild or below. 10% of mice may have a moderate severity due to the appearance of phenotypes whilst still needed for breeding.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The purpose of this licence is to provide a service supplying high quality, controlled and reproducible mice for genetic studies. Many of these strains assessed for suitability as pre-clinical models. Breeding and then phenotyping (on a separate licence) to ascertain whether that particular mouse line is useful in recapitulating aspects of human disease in a way that is useful for therapeutic development. Some of these lines will be generated through the Genome Editing Mice for Medicine (GEMM) or National Mouse Genetics Network (NMGN) funding from the MRC. These lines will be answering a specific research question that has wider implications for more than one research group, and/or will be generating models that mimic the genetic changes found in human disease in order to better understand newly diagnosed genetic disorders. For many of these disorders the primary mechanism is not yet known, therefore studies in a mammalian systemswill be more appropriate and relevant than studies in-vitro and in-silico. All GEMM and NMGN projects have been individually assessed to examine in-vitro or in-silico approaches and only when these approaches are no longer sufficient for the scientific requirement, will we proceed to use mice.

In terms of the other aspects of this licence, such as quality control of inbred lines and holding centralised colonies of well used mouse strains. These must be done in mice due to the nature of the projects.

Which non-animal alternatives did you consider for use in this project?

At this stage, non-animal alternatives are not considered as this should be done as part of the peer review of projects or on the PPL's that the mice will be moved on to. New mouse lines generated through the GEMM and NMGN programmes will justify the need to use mice as part of the application process, and other lines will be moved on to PPLs with authority to use them and explanation of why animal models are necessary. Where work is only to be carried out on service licences, evidence of peer review and the need to use animals will be sought and recorded as part of the commissioning process.

Why were they not suitable?

Breeding and quality control of inbred lines is essential for all the genetic research carried out at the establishment. For specific GA models, in many cases non-animal models are used alongside or prior to work in mice. Mice are also used to generate cells for use in ex-vivo tissue culture. Mice are only used at a point where they are necessary to adequately answer questions that cannot be studied in other, non-animal systems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Each breeding scheme is calculated based on the information from the strain and the genetic background. As an estimate, based on current work for this licence, numbers have been calculated using C57BL/6J baseline data, since the majority of our work is on C67BL/6J or C57BL/6NTac at present.

1. An example of a standard cohort breed:

15 homozygotes needed of each sex. Taking into account average litter size, neonate mortality of C57BL/6J mice and non-productive matings. 28 heterozygous females will need to be set up in 14 trio matings, to produce 160 pups.

15 homozygotes of each sex, 30 homozygotes in total.

Probability of getting one homozygote from a heterozygous intercross is 1 in 4. Therefore 120 mice need to be weaned to get a better than 50% chance of generating the required number of homozygotes.

Average neonate mortality of 25% for C57BL/6J means that 160 mice need to be born for 120 mice to reach weaning.

An average litter size of 7 means 23 litters are needed to generate 160 mice.

For cohort breeds that are set up for only a short period of time ~15% of females do not get pregnant. To compensate for this, 28 females will be mated in 14 trios.

The breeding step to generate the females and males needed for this breed will also be calculated and in this example (using the same breeding data but different ratios depending on the cross) will require a total of 149 mice. Therefore for the two steps of this breed, we will use 309 mice.

If we estimate we will do 50 similar breeds a year for 5 years that is 77250 mice.

2. In addition to cohort breeds, it will be necessary to keep a proportion of lines in a breeding cycle to supply future cohorts or until phenotyping is complete. This type of breed will generate 40-50 mice approximately every 5 months. We estimate we may have 30 of these lines at any one time, approximately 18,000 mice over 5 years.

3. Hold and supply of colonies for use by multiple groups will use approximately 200 mice per colony per year. Holding 12 such colonies would use 12,000 mice over 5 years.

4. Breeding mice for establishing, archiving and QC of genetic alteration and initial welfare assessment will use 50-80 mice per line. For 50 lines a year over 5 years this is 16250 mice.

5. Genome editing GA line that are in intermediate phases of generation requires between 2-5 sessions of micro-injection, each using approximately 20 heterozygous females. This means that between 110 and 260 mice need to be generated per line, an average of 185 mice. Approximately 10 lines will be bred per year for this purpose. 9250 over 5 years.

6. Finally, the genotyping of inbred lines to ensure genetic integrity is carried out on ~200 mice per month which will form the future breeding stock of the inbred colonies. This equates to 12,000 mice over 5 years.

Overall, this equates to 144750 mice over 5 years, approximately 30% of these will be wild type.

This is an estimate based on current knowledge of future work, complex breeds and different genetic backgrounds may change this significantly, therefore the total numbers of animals needed will be under constant review.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We calculate individual breeding strategies taking into account several factors. Including up to date breeding data such as litter size, mortality and fertility, from inbred lines, and where possible, on individual GA strains.

Binomial distribution statistics are used to assess viability of strains.

The design of each experiment is discussed at the outset with members of the Scientific management team. The sample size and the justification for that are discussed, as well as how to generate the cohorts using the fewest mice possible. For example, if the cohort can be split into the batches that can be used more than 7 weeks apart, the breeding stock can be reduced by ~50%.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All lines generated as part of the GEMM project are freely available to the scientific community so are sometimes shared between several groups.

In cases where the viability of the line is unknown, a smaller pilot breed will be carried out in the first instance to ascertain whether there are any unexpected welfare concerns. Statistical analysis shows that we need a minimum of 28 pups to make a reasonable assessment of viability.

For single allele breeds, tried and tested strategies will be used for the most efficient breeding. For more complex crosses, often several strategies are considered and assessed for efficiency in terms of timing, mouse numbers and to reduce welfare impacts. The selected scheme may not always be the breeding scheme that produces the least mice overall, but the scheme that produces the most relevant biological controls and then breeding those as efficiently as possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All genotyping will be done on this licence using ear biopsy, which is also used as a method of identification (except in <1% of cases where a second biopsy is needed). This should cause no more than slight and transient pain and have no healing difficulties.

With the exception of non-schedule one killing of embryos, all other procedures will be carried out under terminal anaesthesia.

GA models will be bred in a way that causes the least harm to the animals, for example, if when carrying homozygous mutations, a mouse strain has a harmful phenotype, we will avoid using homozygotes for breeding and instead use heterozygotes. It is not the scientific intention for any mice on this PPL to suffer from adverse effects, in fact that opposite is true, that mice used for breeding need to be healthy. Mice bred for cohorts will be moved on to PPL with authority to have mice with those adverse effects before the adverse effects become overt. In the case where these adverse effects are present before mice can be moved to another licence, or in breeding stock that, specific justification will be detailed in the protocols.

In some cases it is not possible to breed without some adverse welfare as an impact of the genetic alteration. In these cases stringent humane endpoints will be in place and if possible, breeding limited to an age before the mice become unwell.

Additionally, a large proportion of lines on this licence will be new, or bred in specific combinations for the first time. In these cases, it is not possible to know what the adverse effects will be. Specific humane endpoints will be put in place based on an extrapolation of the symptoms in humans and predicted adverse effects in mice. For example, where humans have a neurological disorder with movement abnormalities, we might expect tremors or gait abnormalities in mice.

Why can’t you use animals that are less sentient?

Mice used on this licence have to be of an age that is suitable for breeding.

Projects will be carried out on this licence when it is necessary to assess the gene function in a mammalian system. The ability to modify genomes in a complex way is more advanced in mice than in other species, although simple modifications are now possible across other species due to the advent of CRISPR/Cas9 technology. Additionally, and critically for genetic studies, there are a large variety of mouse inbred strains in which a genetic modification can be assessed in the context of a standardised genome. Such inbred strains are significantly rarer or not available for the majority of species. Mice have a wealth of baseline data from which to draw comparisons with, both at a phenotype and genetic level. For bespoke breeding projects not part of large funded programmes (e.g. IMPC, GEMM and NMGN) evidence of peer review and the necessity to use mice will be required prior to breeding on this service PPL.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The majority of procedures carried out on this licence will be ear biopsies and >80% of the time this will be done in conjunction with an ear clip taken for identification.

Other procedures are carried out under terminal anaesthesia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The establishment has full AAALAC and ISO (9001:2015) accreditation. To conform to these standards we must ensure a high level of quality control on all aspects including husbandry and administrative processes.

For terminal procedures routes and volumes for administration of substances are taken from LASA guidelines.

All procedures, including husbandry tasks, are carried out to strict standard operating procedures.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The PPL holder and managers working on this licence will attend national and international conferences focussed on the 3R's in animal science such as the 3Rs symposiums, and the national NC3R's meeting. As well as IAT, LASA and similar international conferences where they will attend talks, give talks or run and/or take part in workshops focussed on increasing reproducibility, reducing animal numbers and refining practices. The PPL holder will also attend more specific disease based conferences to gather information on replacement in that specific field.

Developments in the 3R's are a fixed agenda point at every stock meeting to promote the sharing of ideas between the research group and the animal house staff.

13. Information Encoding for Cognition in Mammalian Brain Circuits

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

neuroscience, dementia, therapy, circuits, information processing

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how brain circuits give rise to cognitive processes such as memory, how they dysfunction during neurodegenerative disease, and how they are affected by therapeutic treatments.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Dementia, a term describing a range of progressive neurological disorders which result in impairments to cognitive function, currently affects over 850,000 people living in the UK, and around 50 million worldwide. The most common form of dementia is Alzheimer's Disease (AD). While we know much about the molecular changes that occur in Alzheimer's Disease, such as the build-up of plaques of amyloid protein outside cells, and tangles of tau protein inside cells, we still have a limited understanding of how this disrupts brain circuitry and leads to cognitive degradation. Here we will study how the cognitive functions (such as spatial, episodic and working memory) that are impaired in AD arise from the activity of brain circuits in normal (unaffected) brains, and how they degrade during the progression of AD. As well as improving our understanding of aspects of brain function that are important to all of us, this will enable us to characterise how new treatments affect cognitive neural circuits – providing important information, not otherwise available, that will help to direct research on therapies for AD towards those that will have greatest effect, leading to enhancement of quality of life for dementia sufferers.

We need to use live animals for this work because cultured cell lines do not show AD pathology, and we cannot perform behavioural tests in cell cultures or brain slices. We also perform in vivo work because we want to test the effects of drugs in the brain to demonstrate that they work, and to see if there are any secondary effects, which could be harmful to patients.

What outputs do you think you will see at the end of this project?

Outputs will include (i) publications describing contributions to our understanding of how brain circuits support cognitive operations, how they are affected by neurodegenerative disorders, and how they can be repaired by therapeutic strategies; (ii) datasets collected in the course of our experiments, which may be of wider applicability for understanding brain function and dysfunction; (iii) technological advances made in the course of these experiments which improve our ability to carry out neuroscience experiments of this type.

Who or what will benefit from these outputs, and how?

These outputs will benefit:

Starting immiediately, neuroscientists studying the basic mechanisms underlying cognitive function – who will benefit from information presented in our scientific papers, which will allow them to make further progress on understanding brain function.

During the project, neuroscientists studying neurodegenerative disorders – who will benefit from information presented in our scientific papers, which will allow them to make further progress on understanding the mechanisms of dysfunction in brain circuits. In particular, our data will inform new developments of therapies for Alzheimer's Disease.

Over the longer term (beyond the project duration), clinicians who work with patients suffering from neurodegenerative disorders will benefit from the above advances in our understanding of brain mechanisms of neurodegenerative disease, through having new treatments enter clinical trials.

Over the longer term, patients will benefit from new treatments for neurodegenerative disease, by having an improved quality of life; this will follow indirectly from our research, as the results are translated into the clinic. The wider population will then benefit from the increased productivity of a large and growing sector of society who would otherwise require care.

How will you look to maximise the outputs of this work?

We will use a number of approaches to maximise outputs:

Collaboration with specialists in techniques beyond the core expertise of our laboratory (such as molecular and cellular biology, drug development, etc) will maximise the pace and breadth of research progress, and provide additional opportunities (e.g. access to newly developed mouse disease models and drugs), maximising outputs.

We will collaborate with pharmaceutical partners to test new drugs using our approach, thus ensuring that outputs can have immediate effect on therapeutic development.

We will use outreach to patient groups to help disseminate knowledge of our work, as well as to incorporate patient views at the earliest stage of research to help direct our efforts.

We will use a variety of approaches to disseminate our knowledge, including the traditional routes of journal publication and conference presentations, but also including social media threads, podcasts and interviews.

We will make our datasets freely available in an online database (such as zenodo) at the point of publication, which will allow others to make best use of our data, potentially resulting in additional publications. We will publish methodological papers in addition to papers describing our scientific results, in order to help move the whole field forward and increase the rate of progress.

Species and numbers of animals expected to be used

- Mice: 4500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice of all life stages. We use mice because of the availability of transgenic mouse models of neurodegenerative disorders, and because it is the mammalian preparation in which genetic tools are most developed. In some cases we will need to begin procedures prior to birth, in order to introduce DNA into developmentally defined cell types, whereas other procedures will begin in juvenile or adult mice. Experiments will be typically be performed on mice from young adulthood through to old age. We need to do some experiments in old mice, as we are studying neurodegenerative disorders which progress with age.

Typically, what will be done to an animal used in your project?

A typical mouse in this project will undergo surgery under anaesthesia, in which a dental drill is used to remove a small piece of bone over the cortex, a solution containing a virus is injected into the hippocampus, a glass imaging window is implanted into the cortex down to the level of the corpus callosum, allowing imaging of the underlying hippocampus, and a head-fixation post attached with dental cement. The wound margin is closed up and the animal allowed to recover for a period of several weeks. After this, the mouse begins behavioural training by being habituated to handling and to head restraint through clamping the head-fixation post to the behavioural apparatus, initially for brief periods (minutes) and then longer periods. Behavioural training will occur under conditions of water restriction, in which on weekdays the animal receives its normal fluid intake only in behavioural training sessions, with body weight carefully tracked and welfare monitored. During the behavioural experiments, the mouse is able to explore a persistent visuotactile environment consisting of a carbon- fibre chamber floating on an air-bed; as it explores the environment, the chamber moves relative to the head-fixed mouse. Initial behavioural sessions will involve familiarisation to head-fixation under the microscope; in subsequent sessions, the mouse will explore and make navigational decisions within a maze. While the mouse is performing these behavioural tasks, we will monitor brain activity using a laser-scanning fluorescence microscope (laser scanning of brain activity is imperceptible and, at the intensities used, causes no harm to the animal). Behavioural sessions will last for approximately 60 minutes, and be carried out over the course of several months, in order to track changes in brain circuits. These experiments will be performed in both transgenic mice (which develop amyloid plaques in the brain over the course of their lifetime) and wildtype control animals. As well as using mice genetically altered to produce amyloid pathology, we will also use mice genetically altered to express fluorescent proteins in particular cell types in the brain. This is a non-harmful phenotype.

In other experiments, time-pregnant female mice arrive from a commercial breeder and are housed for a 1-week acclimation period. After one week the ventricles of embryos are injected with DNA via a micropipette through the uterine wall of the pregnant mouse (in utero electroporation) under anaesthesia. The pregnant mouse is allowed to recover and monitored. After the surgery the pups continue to develop, are born, and continue to develop normally.

Another typical mouse (expressing fluorescent proteins in subclasses of neurons, and produced by either transgenic mouse breeding or by in utero electroporation as described above) will be placed under non-recovery anaesthesia in an isoflurane chamber. Once twitch reflexes are abolished, the mouse will be quickly removed from the chamber and killed, in order to extract fresh brain tissue for in vitro experiments.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect our animals to undergo anything beyond moderate suffering. Some animals will be genetically altered to induce amyloid pathology in the brain. Although this is expected to induce cognitive impairment, this does not in itself lead to suffering. Animals will usually undergo a surgical procedure (always under general anaesthesia) and will be closely monitored and provided with pain relief before and after surgery. Animals will have restricted access to water, and may experience thirst. Thereafter, animals will learn to perform simple tasks for reward, that are mentally stimulating. Animals may remain in behavioural assessment for up to several months. Rarely, in less than 5% of cases, complications can occur including weight loss and failure to recover from anaesthesia. However, animals will be constantly and closely supervised by trained individuals. If any animal is deemed to be suffering, they will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)

Expected severity

- Non-recovery 60%
- Mild 20%
- Moderate 20%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals in this project as it involves investigation of the neural mechanisms underlying cognitive processes such as memory and perception. To understand these processes (and dysfunctions of them) requires measuring signals from real brains which exhibit such phenomena.

Which non-animal alternatives did you consider for use in this project?

We considered the use of three-dimensional stem cell (brain organoid) cultures, and the use of computer modelling.

Why were they not suitable?

Brain organoid cultures allow us to assay some aspects of spontaneous neural activity dynamics, however as they do not possess sensory inputs or motor outputs, and do not exhibit cognitive phenomena such as memory or perception, it is not possible to use them to replace all of our animal work. We will make use of such organoid cultures to complement the in vivo animal work and assist in translation to human genetics. We will also make use of computer modelling, however this requires animal experiments to constrain parameters and validate predictions, and thus does not replace animal work.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

A maximum of 4000 animals are required for breeding (and 500 for in utero electroporation) in order to generate the maximum of 2500 mice that would be undergoing scientific experiments. Of these mice undergoing scientific experiments, many (maximum of 1500) will be used for in vitro brain slice imaging experiments; this number is determined by the number of slice experiments that can be done in a laboratory of our size, which limits the amount of science that can be done. The remaining 1000 experimental mice are used for in vivo brain imaging and behavioural experiments. The number of mice needed for these experiments has been determined by careful statistical experiment design, based on data from pilot experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used modern statistical experimental design techniques to determine appropriate experimental structure and sample sizes, in order to ensure statistically valid conclusions while using the minimum number of animals, with a particular focus on the number of

animals used for brain imaging during cognitive tasks, the part of our project with most consequence for individual animal welfare. Our statistical design approach, although done from first principles, is very similar to the approach taken by the NC3Rs Experimental Design Assistant.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We follow advice provided by The Jackson Laboratory for optimal breeding and colony maintenance protocols for each individual strain, ensuring that production of experimental animals is optimised. We will use pilot studies to validate techniques and determine experimental design parameters. In particular, by using inter-individual variation in behavioural performance in relevant tasks as a proxy for variability in the large neural populations recorded, we will reduce the number of animals needed to establish these parameters. We use computer modelling where possible to extrapolate results from animal experiments, reducing the number of animal experiments that need to be performed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice. Mice are a very good animal model for our purposes, because their brains are similar in terms of circuit structure to human brains, and because we can use genetic technologies to mimic human diseases and probe circuit mechanisms. We at all times strive to minimise harm to animals, performing experiments under terminal anaesthesia except where it is necessary to simultaneously measure brain activity and behaviour. Animals are constantly and closely supervised by trained individuals and advice sought from the veterinary team if there is any cause for concern. If any animal is deemed to be suffering beyond the limits specific in the license, it will be humanely killed. Overall, we use anaesthesia, analgesia, and humane endpoints to limit suffering.

Our scientific aims require recording neuronal activity from large populations of neurons deep in the brain while mice are performing many trials of a cognitive task. Surgical implantation of an imaging window is the only way to achieve this. Head-fixation is required for two-photon microscopy (which is needed to distinguish individual cells in three dimensions in tightly packed brain circuitry). Our approach of training mice to perform tasks by exploring an environment (virtual or real-world, as in our apparatus) while able to

run “in place” on a floating platform has been shown to improve habituation and lead to lower stress on the animal, in comparison to tasks where head-fixed animals cannot move, and thus is we believe the most refined approach available.

We will use transgenic mouse models of Alzheimer’s Disease. We have been using the 5xFAD model (and will continue using this to finish off projects currently underway). During the course of this license we will begin using the APP NL-G-F knock-in model, a more refined model that expresses amyloid precursor protein at wild type levels, as opposed to the over-expression of 5xFAD and other earlier models. Earlier AD models such as the APP23 model showed some epileptic activity resulting in 25% loss of animals due to seizures. 5xFAD mice show sub-clinical epileptic activity (as indeed do some human AD patients) but with a very small fraction of mice lost to seizures. Neither epileptic activity nor seizures have been observed in APP NL-G-F mice. We thus see the APP NL-G-F model as the most refined, with the 5xFAD model also being more refined than previous models, while also providing important information relevant to clinical AD patients.

Why can’t you use animals that are less sentient?

We are studying the neural mechanisms of cognitive processes such as memory, and how they degrade in neurodegenerative disorders such as Alzheimer’s Disease. This requires use of animals at the adult life stage. We consider that mice have the minimal level of sentience to study cognitive processes of relevance to the human condition.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will work to continuously refine our practice in animal procedures in conjunction with veterinary and animal services staff. This may include improvements to monitoring, with the potential for technological advances to make improved automated homecage monitoring feasible, and we will continuously work with veterinary staff to maintain best practice in post-operative care and pain management. The NC3Rs Working Group on High-Yield Behavioural Techniques has already yielded some improvements in training of animals, and we will work with colleagues to continuously refine training procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3Rs website provides a wealth of published best-practice guidance which we will follow. This includes documents on environmental enrichment, the use of rodent grimace scales to assess pain, housing and husbandry, animal handling, anaesthesia, etc. Of particular relevance will be the publication from the NC3Rs Working Group on High-Yield Behavioural Technique, which is soon to release a White Paper directly relevant to this project.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am a member of the NC3Rs Working Group on High-Yield Behavioural Techniques, and in the course of the project, will be working with this group on the publication of a White Paper reflecting the state of the art in 3Rs relevant to this project. I will continue to engage with this group and the NC3Rs following the publication of the White Paper, and follow complementary work emerging from NC3Rs activities, and the broader scientific literature, on animal welfare. We will discuss 3Rs issues regularly during our laboratory group meeting, in order to continuously implement advances in 3Rs throughout the project.

14. Repairing the Damaged Brain after Traumatic Brain Injury

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

brain injury, mild traumatic brain injury, neuroscience, axon regeneration, neuroprotection

Animal types	Life stages
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to establish a model of mild and repeat mild traumatic brain injury (TBI) and use these models to understand what happens to the brain after a traumatic brain injury, why the brain fails to repair and regenerate after injury, with a view to developing therapeutic agents to counteract this and ultimately preserve function.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In the UK alone, there were almost 160,000 admissions to hospital with brain injury (including traumatic brain injury (TBI)) in 2016-2017, an increase of 10% since 2006. Men are 1.5 x more likely to be affected with brain injury but admissions for brain injury in females has risen by 23% since 2006.

The main cause of brain injury is motor vehicle accidents, followed by falls and interpersonal violence and increasingly, sports-related injuries. Brain injury costs the UK economy £15 billion per year and is a significant personal burden to the individual, causing immediate symptoms such as headache, speech problems and dizziness but long-term problems such as sensory deficits, sleep problems, memory and mood changes and eventually mental health issues. Cognitive deficits occur but take several months to develop, however, visual problems occur more quickly, including blurred vision, double vision or loss of peripheral vision also occurs after a TBI and this is something we are interested in. Non-invasive imaging methods exist that can be used to scan the eye and determine the extent of brain injury. Brain injury can also cause chronic inflammatory changes which eventually go on to cause mental health problems. For example, there have been some high-profile cases of professional Rugby players in the UK that have been diagnosed with early onset dementia as a result of brain injuries caused during sport. One in five people who suffer a TBI go on to develop mental health problems and so TBI has a multitude of consequences. Currently, there are no known therapeutic agents that counteract the problems associated with brain injury nor are there strategies that promote axon regeneration after injury and the loss of brain function that ensues, leaving an urgent medical need for effective therapies. We wish to identify and test therapeutic targets and agents that will promote the regrowth of axons, counteract the negative effects of injury to the brain and ultimately preserve/promote its useful function.

What outputs do you think you will see at the end of this project?

The primary outputs of this work will be to gather new knowledge on what happens to the brain after a TBI, what goes wrong and what processes cause the eventual long-term changes that lead to dysregulated neuronal functioning. We wish to understand the metabolic changes and cellular changes that occur to neurons and their supporting cells of the brain that causes the eventual damage to nerves, scarring, cell death, oedema and how we can best use this knowledge to derive therapeutic strategies to overcome repair and regeneration in the brain. We also wish to know what changes occur to the visual system after TBI as visual problems are frequently reported by patients. We will do this through non-invasive eye imaging techniques and analyse the nerves and eye once the tissues are harvested.

As a consequence of the information we will obtain on new pathways and signalling molecules, we will also identify new therapeutic targets to not only prevent the negative consequences of injury to the brain but also to develop strategies aimed at promoting the

repair and regeneration of injured nerves. Moreover, we will determine if imaging the eye can be used as a surrogate of brain injury. A major potential benefit from the work proposed will be the acquisition of new knowledge for dissemination in peer-reviewed journals. Functional outcomes after brain injury are poor at best and presently only palliative drug treatments are available for patients. At the moment nothing is available for patients that can reverse the damage to the brain caused by the injury. Therefore, there is an urgent unmet clinical need for new therapies aimed at enhancing functional repair responses of damaged brain.

Specific identifying outputs will be to publish the findings. This is important for the development of the project but also to provide a knowledge base for other academics working in this field.

Other product outputs will be to support the development of the new and existing intellectual property which will allow us to facilitate translation into a clinically and commercially viable proposition.

Who or what will benefit from these outputs, and how?

In the short term, the benefits would be to provide new knowledge of what molecules and signalling pathways are affected in the brain after a TBI and identify potential targets where drugs can be developed to improve outcomes in future studies.

In the longer term this work has the potential to identify new therapeutic targets that could be used to develop new therapeutic intervention for those afflicted with TBI. There is currently no treatment that reverses the pathological effects of brain and coincident eye injury and the functional loss that occurs as a result is permanent. Our discovery strategies would provide key information on the pathological processes after brain injury, allowing us to identify opportunities to test and develop therapies aimed at reversing or protecting against the negative effects of injury. As a result, we will identify opportunities to preserve or repair brain function.

Examples of other potential beneficiaries of the success of this work are spinal cord injured or stroke affected patients who will benefit through the development of new treatments that are neuroprotective, pro-regenerative and anti-scarring, since the same pathological processes after brain injury also occur in these groups of patients. Additional beneficiaries could include health care providers, particularly the NHS.

The UK economy spends £15 billion treating brain injured patients every year and represents a significant financial and personal burden. Effective therapies that protect brain from death and promote its repair and regeneration, if successful, would relieve the pressure on the NHS and significantly reduce the personal and financial costs of treating brain injured patients.

How will you look to maximise the outputs of this work?

We will maximise the outputs of the work by collaborating with colleagues and companies working in this field to maximise the use of the data we obtain. We will rapidly disseminate the outcomes of the tests, whether negative or positive, to inform the academic community and support other researchers developing technologies in this area. We will seek partnerships with appropriate pharmaceutical companies when potential therapies have been identified, giving us the potential to translate our therapies into the clinic for patient benefit.

Species and numbers of animals expected to be used

- Rats: 1040

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use adult rats for all of our experiments since they more closely mimic the human molecular responses to brain injury than mice for example. We cannot use lower sentient species like zebrafish as they regenerate their brain after injury. As in the human, the adult rat brain also has a poor intrinsic capacity to spontaneously repair and regenerate its central nervous system and hence is invaluable in finding new therapies for adult human brain injury.

Typically, what will be done to an animal used in your project?

All animals will receive pre-emptive pain relief as a precaution since there is no evidence of pain such as headache, and undergo a regulated procedure to cause a mild TBI (mTBI). Some animals will undergo a repeat mTBI at 3 days after the first injury to assess the impact of repeat mTBI. This is regarded as a moderate severity injury. All animals will receive pain relief prior to undergoing mTBI and after the procedure, as a precaution, to minimise any potential discomfort that may be experienced.

Animals will then receive injections of therapeutic agents and undergo non-invasive tape sensing and removal sensory test and ladder crossing locomotor function tests once weekly for up to 12 weeks.

Animals will then be killed humanely before collection of tissues for histological/protein/mRNA analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Mild TBI: We expect no discernible physiological changes after injury. However, only if challenged in sensory and locomotor tests will we be able to see minor deficits in neurological function affecting their fine movement and sensation. Therapeutic substances/biomarkers are carefully selected from published literature or pilot studies to cause as little discomfort as possible and any discomfort will be transient.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All animals will experience a moderate severity injury.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Whilst some elements of brain injury can be modelled in cell or brain slice cultures, the complex, clinical picture and interaction of the whole-body systems, including in particular the immune and nervous systems cannot all be currently modelled in cell-culture or computer-based models. The use of live animals is therefore unavoidable and essential for discovery science, drug discovery and to demonstrate the activity of drugs in a situation relevant to the human condition. Neurons are not present outside the animal kingdom and so an animal is required. Only mammals have a sufficiently developed immune-system to readily compare to humans, and rodents are the animals of lowest neurophysiological-sensitivity required to achieve the scientific aims. Zebrafish or other lower sentient animals cannot be used since these species spontaneously repair injuries to their brain and are therefore not representative of the human condition. Therefore, there is no feasible alternative that can entirely replace the use of a living animal that would allow our objectives to be met. However, we will use in vitro and ex vivo work to inform our animal studies.

Which non-animal alternatives did you consider for use in this project?

There are currently no alternatives to animal work for the brain injury model.

No cell culture-based models exist that encompass all of the aspects of disease for any of the models described in this project. However, individual aspects will be modelled in vitro and ex vivo. For example, we regularly use in vitro neuronal and glial cell cultures to detect therapeutically useful molecules in terms of neuroprotection and axon regeneration to then take forward into animal studies.

Why were they not suitable?

The fundamental reason why the use of animals is required is to understand these complex processes that occur in a damaged brain, which at present no in vitro methods can model the complexities of the systems involved in this condition. It is almost impossible to use primary cells to culture all of the different types of cells in the brain since they require different growth mediums and factors for survival. Indeed, the reason why many new drugs fail between cell culture and in vivo studies is in the inability to full recapitulate the in vivo environment. Technologies are being developed to address this gap, including the development of 3D cultures. However, none of these model systems are yet able to phenocopy the integration and interplay between the numerous cell types that constitute brain injury.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers of animals have been based on pilot data, in-house and published data.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used the NC3Rs EDA system to calculate animal numbers to be used for this project with calculations setup to achieve a statistical significance of α $p<0.05$ with a power of 0.8. Whenever possible, we used our own published data to feed into the EDA system to generate samples sizes.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will seek to refine protocols, such as the development of other quantitative measures for injury such as associated biomarkers that can predict potential injury severity and recovery and imaging methods such as optic coherence tomography to non-invasively monitor mTBI changes that can be detected in the eye, which will facilitate “reduction”.

Experiments will be planned so that they can be published in accordance with the ARRIVE 2.0 guidelines.

Wherever possible, we will use archived samples and share control groups across different experiments.

As part of good laboratory practice, we will write a protocol for each experiment including: a statement of the objective(s); a description of the experiment, covering such matters as the experimental treatments, the size of the experiment (number of groups, number of animals/group), and the experimental material; and an outline of the method of analysis of the results (which may include a sketch of the analysis of variance, an indication of the tabular form in which the results will be shown, and some account of the tests of significance to be made and the treatment differences that are to be estimated). We will make appropriate arrangements to randomly assign animals to experimental groups and blind studies.

At the end of the experiment, we will harvest the maximal possible number of tissues and biofluids. Tissues not immediately analysed will be archived and will be made available to other researchers working on similar questions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Due to the nature of the scientific question, the only appropriate model is the mTBI model in rats. In the human condition, most mTBI patients develop only have transient headaches and so do not seek medical help as his resolves over a few days. However, the biological consequences can go on from minutes to years after a mTBI. Animals do not show overt outward signs of TBI and hence it is the most refined method of injury as we use lighter weights, short drop height, attach a protective metal disc to prevent injury to the skull and use a thick polyurethane sponge to not only absorb the forces delivered by weight drop but also to protect against contrecoup injury. In addition, we will provide peri- and post- operative pain relief despite there being no evidence of pain, such as headache, as a cautionary measure. The model we will be using has been refined over 20 years by our colleagues in Italy and we will make every effort to ensure the approaches cause the least possible distress, suffering or harm.

Why can't you use animals that are less sentient?

We cannot use less sentient species (e.g. zebrafish) for this work, because unlike mammals, they are able to repair injuries to the brain spontaneously. Rats will be used for our experiments since they share similar pathophysiology to humans after injury to the brain. We do not use mice to model the human TBI since the responses in the CNS of a mouse are generally further away from the responses in rat to TBI. Some in the field have argued for larger animal models to be used. For example, rabbit, sheep, swine and monkey models have been proposed, however, all of these models require a craniotomy and have high levels of mortality. Neither of these parameters are common in human TBI and hence are not representative. Moreover, there are only a handful of studies using these models and little is known about the pathophysiology of TBI in these models compared to the rat which has received a lot more attention. Therefore, our rat model is the best model for use in understanding what happens after a human mTBI.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The weight-drop mTBI model has been refined extensively. For example, a stainless- steel plate is glued to the skull prior to weight drop. This prevents injury to the skull and causes a generalised head injury as experienced by humans. The thick polyurethane foam pad that the animal is placed on prior to weight drop prevents contrecoup injury after the weight drop since it is able to absorb the energy and prevent whiplash injuries occurring to the rat.

The use of surrogate biomarkers in peripheral blood and CSF has also reduced the need for increased animal usage since these samples can be harvested from the same, live animals and hence additional injury stratifying parameters can be developed. In addition, imaging the back of the eye has allowed changes in the eye to be used as surrogate markers of TBI. All of these additional measures reduce the need for additional animal experiments.

Non-invasive imaging of the eye is a potential surrogate marker of brain injury since neurons in the eye are also affected after mTBI. Hence, we may be able to develop new knowledge of how the eye can be used to both diagnose and prognosticate mTBI.

Some of the potential therapeutic agents can be evaluated and optimised in vitro prior to in vivo application. Others will be optimised in small pilot experiments with drug doses based on published literature. We keep our experimental time points in longitudinal studies to a minimum and use archival control results where possible. Multiple analyses are done on harvested tissues. We use the minimum number of interventions and minimal volumes for drug delivery during experiments and continually seek methods to reduce these by studying alternative drug delivery strategies. Although there is no evidence of pain such as headache, all animals will receive pain relief prior to and after the procedure as a precaution. They will also be picked up using refined handling techniques to minimise distress and the technical staff are well versed in recognising the signs of distress in these

animals as they have many years' experience in handling and caring for our injured animals. We use bespoke welfare sheets that include body conditioning scores to monitor animals post-operatively. All of these refinement steps significantly reduce the animal welfare burden.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Prior to all experiments we will consult the PREPARE guidelines checklist to ensure that valuable data will be generated in the experiment.

Experiments will be conducted in accordance with the guidelines published by the Laboratory Animal Science Association (LASA).

The resulting data will be published in Open Access Journals wherever possible and in accordance with the ARRIVE 2.0 guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed of advances in the 3Rs through attendance of seminars and conferences, as well as discussions with the named veterinary surgeon and the named animal care and welfare officer.

We will review each experiment on completion to determine any refinements that can be applied to future experiments.

Continued review of the scientific literature will be undertaken on a regular basis in order to identify any newly emerging technologies and models that could be potentially adopted in order to replace in vivo animal use.

We will also stay up to date with guidance published by LASA on the most refined experimental methods. We are already signed up to receive the NC3Rs newsletter and will attend local events such as conferences and follow advice in webinars hosted by NC3Rs.

15. Genetically Altered Rodent Generation, Breeding and Maintenance.

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Physiology, Genetic Manipulation, mice

Animal types	Life stages
Mice	adult, embryo, pregnant, juvenile, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The creation, breeding and supply of novel and established lines of Genetically Altered (GA) mice. A GA mouse is a mouse that has had its genome altered through the use of genetic engineering techniques. Genetically Altered mice are commonly used for research as animal models of human diseases, and are also used for research on genes. Mice will be of high health status and defined genetic quality for use in research projects at this and other establishments. Cryopreservation of such lines provide health and genetic security. Mouse embryos or sperm are frozen in liquid Nitrogen, to avoid loss by contamination, unexpected disasters eg fire in a facility and genetic drift. Genetic drift is where genetic changes happens constantly, even small changes might have an impact on future research and replicability.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is recognised how important genetically altered mice are in understanding the pathophysiology of many disease conditions including cancers, cardiac and metabolic diseases, as well as improving our knowledge of basic physiology. Creating, breeding and maintaining genetically modified animals we will ensure that such animals generated are produced to the highest standards of health and welfare enabling more reproducible and publishable research. Thus we are able to recruit top quality research scientists.

Cryopreservation provides security against genetic drift and health status contamination whilst meeting the 3R's.

What outputs do you think you will see at the end of this project?

Provision of GA models to researchers, resulting in new knowledge of physiological and disease mechanisms and dissemination via peer reviewed publications

Who or what will benefit from these outputs, and how?

Researchers here and their collaborators, other research facilities who do not have the technical expertise to create GA mice.

How will you look to maximise the outputs of this work?

Peer reviewed publication of the subsequent work on these animals, including encouraging publication of any failures of the approach, collaborations with other institutions.

Species and numbers of animals expected to be used

- Mice: 17700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are a well recognised species for work involving genetic alterations and there are standard protocols, methods and reagents used that have been optimised for this species and acknowledged benefits for use.

Typically, what will be done to an animal used in your project?

The majority of the animals will be used for the breeding and maintenance of genetically altered or mutant animals. Some animals will undergo surgery as follows-

Typically female mice will be mated with vasectomised (sterile) male mice to induce pseudo-pregnancy (in mammalian species, pseudo-pregnancy is a physical state whereby all the signs and symptoms of pregnancy are exhibited, with the exception of the presence of a foetus, creating a false pregnancy).

They will then undergo a minor surgical procedure, under general anaesthesia, to implant previously genetically modified embryos into the oviduct. During this procedure analgesia will be administered. The females will be allowed to recover and give birth. Aseptic technique will be observed throughout. The resulting offspring will have a small piece of ear tissue removed to confirm the genotype.

What are the expected impacts and/or adverse effects for the animals during your project?

Superovulation -There may be momentary pain on 2 occasions when an I. P injection into the abdomen is administered.

Recipients -Surgical: A female mouse will have a small, less than 1 cm incision, to expose the Uterus, enabling delivery of embryos into the uterus. Mice recover rapidly and, in our hands, without complications. Anaesthesia, analgesia and aseptic technique used to prevent pain and infection. -Non- surgical: A female mouse will have a small speculum inserted in the vagina and positioned around the cervix, the catheter is then inserted into the speculum and through the cervix, at this stage embryos are expelled into one of the uterine horns. Anaesthetic will be used.

Vasectomy -Minor surgery from which mice recover rapidly and, in our hands, without complications. A small incision approximately 5mm, is made into the scrotum and the Vas deferens is cauterized to render the mouse infertile. Anaesthesia, analgesia and aseptic technique used to prevent pain and infection.

Breeding of genetically altered animals with mild phenotype (the observable traits eg eye colour is a phenotype). These animals are not expected to show any deviation from normal wild type mice. A

typical breeding female will have 6-8 litters in her lifetime.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority, approximately 60%, of the mice are perfectly normal and will suffer severity which is sub- threshold. Approximately 30% of the mice will have 2 injections into the abdomen which is mild severity. Some mice, approximately 10%, will undergo minor

surgery will have moderate suffering which is expected to be brief and resolve without complication.

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

New technologies are improving the field of genetic engineering in animals and they will allow the generation of new mouse models to be applied in biomedical research. First steps in a scientific project will involve in vitro approaches, the final characterisations and applications will require the use of genetically altered animals. The different animal models will integrate the complete range of molecular, cellular, physiological and behavioural interactions necessary to fully understand how genetic modifications result in normal or abnormal processes

Which non-animal alternatives did you consider for use in this project?

We have an established cell engineering service as a potential alternative to in vivo models in vertebrates.

Why were they not suitable?

In-vitro assays cannot adequately model the complete array of molecular, cellular, physiological and behavioural interactions necessary to fully understand how genetic modifications result in normal or abnormal processes.

Invertebrates are a useful adjunct to animal studies in that large throughput of gene alterations can be screened but the differences in circulatory, neurological systems etc limit their use.

Prior to importing or creating a new strain, consideration will be given to the scientific evidence gathered from in vitro data e.g. receptor binding assays to identify appropriate targets and PCR analysis of gene expression. The breeding method and proportion of affected animals produced will also be considered.

All of these factors will be used to justify the introduction and creation of strains under this licence with advice and discussions taken with the researcher and NVS (Named Veterinary Surgeon) as required

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are predicted, based on analysis of the number of animals used in the last five years to meet the demand of our researchers.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Switching to an IVF (In Vitro Fertilization) -based approach to generate mouse embryos in place of traditional overnight mating's reducing the number of mice used by half.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Using efficient breeding techniques including using the Home Office genetically altered animal breeding guidelines

Switching to an IVF-based approach to generate mouse embryos in place of traditional overnight matings reducing the number of mice used by half

Integrating CRISPR-Cas9, a gene editing technology, which has greatly reduced the numbers of mice required to make a new transgenic strain for many allele types.

Developed our own method to generate high efficiency long single stranded DNA (lssDNA) donors for gene knock in projects.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are a well-recognised species for work involving genetic alterations and there are standard protocols, methods and reagents used that have been optimised for this species and there acknowledged benefits for use.

Embryos, gametes, sperm and ovarian tissue will be collected from donor strains. They may be cryopreserved or used for the following purposes.

- Fresh or frozen embryos and sperm will be used for rederivation of infected mouse strains to improve health status.

- Gametes, embryos/sperm or/and tissue will be archived by cryopreservation for strain storage in support of the breeding colonies.

- Gametes, embryos/sperm or/and tissue will be used to replace a strain for storage where there is no longer a research requirement to avoid wastage from maintaining ‘tick over’ breeding programmes.

- Gametes, embryos/sperm or/and tissue will be used where possible instead of live animals to transfer strains to other establishments within the United Kingdom and abroad

Why can’t you use animals that are less sentient?

It is fundamental that the GA embryos are implanted into a receptive female and she is then allowed to complete gestation. The resulting offspring will be used in research projects for which the whole mammalian body systems are required.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

One of the chief benefits of undertaking this work under a service licence is that all the techniques to be used are undertaken by a small group of highly experienced technical staff which minimises suffering. It also ensures that the highest standards of asepsis are maintained and that appropriate and effective analgesia is always used.

We are looking into using NSET (non-surgical embryo transfer) for some of our projects, but at present NSET is not suitable for the majority of our pipelines as these involve microinjecting 1-cell embryos.

For NSET these would have to be cultured to blastocyst, the problem we experience is that 1 & 2-cell embryos culture well to the blastocyst stage (80 to 90%) but when they are transferred into the uterus the implantation rate is quite poor. Therefore, we feel that surgical transfer of 1-cell embryos is still the most refined method.

We are a part of an NC3R's funded project to develop a genetically sterile male mouse which could replace the vasectomy surgery.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will consult with colleagues across the field and sources such as AWERB

International society for Transgenic Technologies Animal Welfare and Management Discussion Group NC3R's Efficient Breeding Strategy

Institute of Animal Technology

Efficient Breeding of Genetically Altered Animals Assessment Framework(Home Office)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Advice has been sought from NC3R's project manager on the development of this PPL application and will continue to be sought during visits to this facility on a weekly basis.

16. Imaging and Modulating Ocular Immune Responses

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Uveitis, Immunity, Retina, Immunohistochemistry, Intravital

Animal types	Life stages
Mice	adult, juvenile, pregnant, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To increase our knowledge of the role of the immune system in the eye during health and disease employing advanced imaging techniques to allow better diagnosis and treatment of conditions that lead to blindness.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Sight loss is profoundly disabling and affects huge numbers of people across the World. It is progressively clear that abnormal immune responses are implicated in almost every ocular disease, including uveitis, age-related macular degeneration and diabetic retinopathy. Together, these diseases account for over 70% of blindness registrations in the UK and cost the NHS an estimated £3 billion in direct costs and the wider social care system £6 billion per year. Their incidence is projected only to increase as a result of obesity and a rapidly ageing population.

Current methods for the diagnosis and monitoring of these diseases are limited, often requiring surrogate or proxy measures that can result in over or under-treatment. Despite progressively improved treatment options, many patients continue to fail to respond adequately, lose sight or come to harm from side-effects of medications.

There are now huge opportunities to address these unmet needs by deploying advances in ocular imaging and post-mortem tissue analysis technologies.

What outputs do you think you will see at the end of this project?

New information: The proposed work will expand our understanding of ocular inflammation and immune responses in relation to blinding diseases. We will apply novel techniques such as adaptive optics retinal imaging and highly multiplexed immunohistochemistry to reveal previously unknown aspects of pathology. By studying the effects of therapeutic treatments, new pathways to increase their effectiveness or reduce their side-effects could be discovered.

Publications: Following on from our established track record, we will continue to publish our work in international, peer-reviewed scientific journals such as PNAS, Molecular Therapy, eLife and Science Translational Medicine. Wider outputs will include presentations at international and domestic scientific conferences such as Association for Research in Vision and Ophthalmology (ARVO) annual meeting or RCOphth and British Society for Immunology congresses. We will continue to engage with patient groups and research charities to ensure continued engagement and participation in the benefits of the research.

Who or what will benefit from these outputs, and how?

Short-term: Immediate scientific benefits to the research group will be derived from an increased understanding of ocular biological processes and the continued development of research expertise and techniques.

Medium-term (2-5 years): The work is expected to benefit researchers in the fields of ocular disease and immunology, reached through the dissemination of our findings through publication, collaboration and scientific conferences. Discoveries will be shared with the public and patients through outreach activities.

Long-term (over 5 years): This project will benefit medical specialists working in ophthalmology and patients through the development of better biomarkers and monitoring of ocular diseases. A greater understanding or novel discoveries relating to therapies will benefit patients after engagement with clinicians and industry.

How will you look to maximise the outputs of this work?

Collaborations: The impact of our work will be maximised through continued collaborations with scientists across other disciplines. We already have established links with engineering, physics and computer science colleagues who can employ data produced in our investigations to develop new approaches to modelling and image processing.

Dissemination: Our discoveries will be shared at scientific conferences, through scientific publications and public and patient engagement activities. We are committed to publishing unsuccessful approaches for wider public benefit and have in the past published negative results from clinical and scientific studies.

Species and numbers of animals expected to be used

- Mice: 3500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice will be used as the least sentient species for which established models of the ocular diseases exist to undertake the proposed studies and answer our research questions. In addition, mice are the most amenable to the genetic alterations needed to label immune cells. Adult mice will be used as the imaging techniques required are easier to perform at this stage of eye development.

Typically, what will be done to an animal used in your project?

The purpose of the project is to better understand the role of inflammation within the eye in relation to blinding eye diseases and to inform and improve our therapeutic approaches in patients. A proportion of the mice used this project will be genetically altered and bred, however these are not expected to have harmful effects or cause suffering.

Most of the studies will involve the initiation of the disease by injection of substances systemically, into the eye or by damaging the retina with a controlled laser treatment. All of these will be performed under general anaesthesia, so mice do not experience pain or discomfort. The eye diseases induced are mild and not painful, and whilst the animals may experience a degree of sight loss this does not result in alterations to their normal

behaviour, as mice are more reliant on other senses such as smell, hearing, taste and touch.

Typically, following disease induction, the eyes of mice will be examined (usually twice weekly), under general anaesthesia, to monitor the progression of the inflammation. In addition, small blood samples from the tail may be collected and some of the animals will be given treatments aimed at preventing or minimising the severity of disease. This is usually by giving a drug by injection. At the end of the study the mice will be killed using a humane method.

What are the expected impacts and/or adverse effects for the animals during your project?

The disease models used in this project are mild. While some sight loss may occur, it does not cause pain or result in the animals changing their behaviour. Furthermore, in the mouse, vision is a much less important sense than smell, taste, hearing and touch using their whiskers.

Most of the procedures are conducted under general anaesthesia, which ensures discomfort is minimised. However, as a consequence the animals will experience several anaesthetics during the course of the study which can be cumulatively burdensome.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Half the mice will be used for breeding and will only experience the mild severity category. The remaining animals will be in experimental protocols in a moderate severity category as that is the maximum potentially achieved, though for the vast majority it is not expected to exceed a mild severity in practice.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

As the studies use imaging to study dynamic immune responses, a living intact retina, a circulation and viable immune system are required. The only way this can currently be achieved is through the use of animal models. The mouse is the least sentient species that has a relatively comparable anatomy to the human retina and models of ocular inflammation - such as experimental autoimmune uveoretinitis (EAU) and laser injury are already established. The mild visual loss caused by these models is attenuated by the dependence of mice on other senses given their typically nocturnal environment, such as smell, taste, hearing and touch. Their normal behaviour is rarely affected and minimal distress occurs. No alternative animal model has a less severe disease course.

Which non-animal alternatives did you consider for use in this project?

We looked into computational modelling and also at stem-cell and organoid models. We have previously used human stem cells treated to become RPE ocular cells to look at specific responses to cytokine stimulation and this could be used to refine potential candidate immunotherapeutics, but cannot replace all of the experimental approaches required.

Why were they not suitable?

Computational or in vitro models currently are not able to provide the required biological data, given the complexity of studying multiple cell types, cell motility and emergent properties in the full tissue environment proposed in this project. Some of the most advanced models capable of looking at detailed immune cell interactions can only handle three cells at one time.

Stem-cell and organoid modelling is currently at a stage where different cell types can be grown, but the precise anatomy is not retained and combining the many different immune cell types and blood supply required to model an immune environment has not been achieved.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals needed to generate and maintain the genetically altered (GA) mice needed for the study is estimated at 300 animals per year. This figure is based on the number required to maintain the different transgenic lines and supply the mice required for experiments (Protocol 1).

Experimental usage (Protocol 2) is estimated at 400 per year based upon the scientific objectives of current funded projects and equates to around 10 experiments (each with up to 3 replicates) to an average of 30 experiments per year. In estimating group sizes we have undertaken analysis of data from previous experiments and performed calculations using statistical and design packages as detailed below.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Standard online tools and packages were consulted but they largely assume the use of large numbers and parametric statistics, which are often inappropriate for the readouts and assessments of models of ocular inflammation and assessment by immune cell counting. We therefore used dedicated packages such as the Experimental Design Assistant (EDA) from NC3R, which we will continue to use going forwards.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use efficient breeding and allocate males and females to specific disease models based on susceptibility to induction (e.g. EAU with females, laser models with males). Tissue sharing will be continually encouraged, made easier through belonging to a large ophthalmic focused research institute. Open data sharing as per grant requirements and the University data management policy will be prioritised and this will also support computational groups within the department.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mouse models that replicate key ocular inflammatory diseases (e.g. uveitis and neovascularisation or degeneration as seen in age related macular degeneration) will be used. Genetically altered animals are needed for many experiments, although the alterations have no adverse effects on the animals. Care has been taken to select mouse models that induce only mild disease and none are known to be painful or of significant distress in most cases. The associated loss of sight does not impair the animal’s ability to perform its normal behaviour, as mice are more reliant on the other preserved senses. Careful monitoring and assessment of disease will be undertaken using non-invasive

imaging methods conducted under general anaesthesia so involves minimal distress to the mice. The delivery of therapeutics or collection of blood samples will be undertaken using well-established techniques that cause no more than mild transient pain and no lasting harm. We will implement intermittent breeding in line with recent NC3Rs guidance to minimise unnecessary wastage of animals.

Why can’t you use animals that are less sentient?

Mice are the least sentient of animal species to have eyes that are anatomically similar to humans and for which established disease models for ocular inflammation exist. Their immune systems are also recognised as sufficiently similar to man and despite some caveats have been already used to successfully design clinical therapies and drugs.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We continue to refine our methods for the induction and monitoring of ocular inflammation and have published on this before in the scientific literature.

By using non-invasive ocular imaging with developing and advanced methods of tissue analysis, we can avoid the frequency of more invasive sampling such as for blood. We can also extract more data from each animal, reducing the overall numbers required.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We participate actively in 3Rs seminars, presenting our approaches and learning from others in the ophthalmic research community, with weekly seminars at the University and special interest groups at international vision research conferences such as ARVO. We will ensure all our work is compliant with ARRIVE guidelines and consult the latest refinements from NC3Rs and LASA.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our group takes part in continuing education including attending and contributing to 3Rs focused seminars and lectures and through conferences and publications discussed at journal clubs. We will engage with regional NC3Rs programme managers and training programmes offered by the University Biological Services Unit.

17. Developmental Defects Caused by Mutations in Chromatin Genes

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

chromatin, gene regulation, developmental disorders, heart development, brain development

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to understand why mutations in chromatin genes, which should be required in all tissues, affect certain organs more than others (most commonly the heart and the brain).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

More than 100 human genetic disorders are caused by mutations in chromatin genes. From what we understand about these genes, they should function in all tissues. However, when they are disrupted they only cause problems in some tissues whilst leaving others

unaffected. Before we can research treatments that could help patients with these genetic diseases, we first need to understand why they have such different effects on different types of cells. This is important because otherwise potential treatments might cause side-effects by disrupting organs and tissues that were previously unaffected by the mutation.

What outputs do you think you will see at the end of this project?

- 3D images of developing mouse embryos carrying mutations in chromatin genes.
- Atlases of gene expression in heart and brain tissue from these embryos, showing where the mutation has caused other genes to be improperly switched on or off.
- Datasets showing how the mutation has affected the way DNA is folded, packaged and protected inside cells.
- Software tools for integrating these different datasets.
- Research publications that explain the datasets and use them to identify why the mutations have specific effects on heart and brain development.

Who or what will benefit from these outputs, and how?

Researchers working on these diseases will be able to use the datasets to identify genes disrupted by the mutation and target them with new therapies that might help reduce disease symptoms.

Researchers working on the basic biology of the genes we mutate will be able to use these datasets to gain new insights into the mechanisms of those genes.

Researchers working on the genetics of other diseases affecting the heart and brain will be able to use the datasets collected from our healthy controls to examine whether DNA packaging and folding might be a contributing factor to their diseases of interest. They will also be able to use them to identify previously unknown regions of DNA that might be responsible for switching on or off the genes that they are interested in.

Researchers working on the biology of other tissues will be able to repurpose the software tools we will develop for the analysis of their own datasets.

How will you look to maximise the outputs of this work?

Datasets will be released as soon as possible after they are generated (after a maximum of 12 months). Each dataset will be accompanied by a full description of the methods used to generate it and details of the mutations, sex, and age of the mice. An area of the lab's website will be dedicated to cataloguing these outputs and will be advertised in talks, poster presentations, primary publications and through social media. I will also create interactive pages allowing other users to explore the datasets without having to download and reanalyse them. This policy of releasing research outputs prior to primary publication maximises their potential for re-use by other scientists and minimizes the possibility of

other researchers duplicating the same experiments. It is particularly important for research on rare genetic diseases where very little data is already available. We will also publish the details of any unsuccessful approaches to prevent other researchers wasting resources.

Species and numbers of animals expected to be used

- Mice: 11000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The human diseases we are interested in generally cause problems with the heart and the brain during early development (i.e. before birth). We use mice because they have hearts with four chambers, similar types of cells in their brains and because many of the genes involved in forming their hearts and brains are the same as in humans. It is also relatively easy to introduce the types of mutation we are interested in into their DNA. We use mouse embryos because we are interested in the process of their early development in the womb and how these types of mutations cause that development to go wrong.

Typically, what will be done to an animal used in your project?

Most mice will be born carrying "conditional" mutations that will not cause them any health issues. These mice will then be bred in such a way that the mutation becomes detrimental only in the developing embryos. Pregnant females will be humanely killed and tissue taken from the embryos for analysis.

In some cases, we need to administer a substance to activate the conditional mutation in the developing embryos. Typically, this will involve the pregnant female being fed an additional substance either in their diet or drinking water or once per day through a feeding tube for up to five days. The pregnant female will then be humanely killed and tissue taken from the embryos for analysis.

In some cases, we need to administer a substance to be incorporated into the cells of the developing embryo and label them. Typically, this will involve the pregnant female being given an injection under the skin. The pregnant female will then be humanely killed and tissue taken from the embryos for analysis.

Rarely, we may need to combine these approaches, such that the pregnant female is fed a substance via a feeding tube once per day for up to five days and then receives an injection under the skin. The pregnant female will then be humanely killed and tissue taken from the embryos for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals are not expected to suffer adverse effects related to their breeding or genetics.

Animals may experience adverse effects from the administration of substances:

- Injection under the skin of a pregnant female may cause redness or inflammation at the injection site. If this is observed any further injections will be halted and advice will be sought from the vet on an appropriate treatment (e.g. an anti-inflammatory cream). Pain or suffering could last for a maximum of 24 hours, as the animal will be humanely killed if its condition has not improved after this time.

- Injection into the body of a pregnant female may cause miscarriage or reabsorption of embryos. Any pain or suffering will be brief because the animal will be humanely killed immediately in the event of such symptoms.

Animals may experience adverse effects from the substances themselves:

- Animals may experience transient weight loss. Animals will be monitored by condition scoring and any under-conditioned animal will immediately be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice 90% sub-threshold, 5% mild, 5% moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are interested in the processes by which the heart and brain are formed, and how these go wrong in certain genetic conditions. Normal development requires a complex set of interactions between different organs and tissues. These processes and interactions are currently not possible to study in depth using non-animal approaches.

Which non-animal alternatives did you consider for use in this project?

We have searched databases for scientific publications and data pertaining to the genetic conditions that we are interested in. We will continue to do this through the life of the project to ensure that we avoid wasting any animals duplicating experiments already performed by others, or conducting experiments whose hypotheses have already been addressed by others. We regularly download and re-analyse publicly available datasets. Often these come from wild-type mice with no mutations mimicking human conditions, but these are useful to design our experiments and ensure that we are targeting the appropriate cells. This avoids unnecessary use of animals in pilot studies.

As alternatives to animals in this project we have considered the use of human patient tissue, culturing of stem cells derived from human patients, introducing mutations into normal stem cells to study how this affects the formation of brain and heart organoids and the use of cell lines derived from heart or brain cell types.

Why were they not suitable?

The genetic diseases we are interested in studying are rare, so human tissue is very hard to obtain. More importantly, the processes that we are interested in take place during pregnancy, so we would only be able to obtain human tissue samples years or decades after the period of development we are interested in.

Methods for growing cells in culture to form structures that functionally resemble the heart and the brain are improving every year, these are called cardiac organoids or brain organoids respectively. However, none of these techniques is perfect and all of them are only useful for exploring particular processes or types of cells. Because so little is known about the diseases we wish to study, it is impossible to judge whether any of these techniques might be suitable. Our approach instead is to conduct the early stages of this project in whole animals, then hopefully to progressively move to these alternative approaches as we understand which particular cell types we should focus on to better understand the disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We first make a list of the experiments needed to address our aims. We then use available tools to estimate the number of embryos required for each experiment, such that we use the minimum number required to obtain robust and statistically meaningful results. Because we know the average number of embryos carried by each pregnant female, we

can calculate the number of female mice needed, and the number of male mice needed to breed with them. Finally, we calculate the number of breeding pairs required to generate the male and female mice to be used in each experiment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the NC3R's Experimental Design Assistant to ensure our experimental designs are robust and to identify appropriate methods for analysis of the data, which is an essential first step to minimising the number of animals required for an experiment. We also refer to the PREPARE guidelines published by Norway's 3Rs centre Norecopa whilst designing each experiment. All experiments will be conducted and records kept such that publications arising from this project will comply with the ARRIVE guidelines

2.0 maintained by the NC3Rs.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For each experiment, we compare all possible ways of combining male and female breeding animals to determine the approach which minimises the number of animals required. This includes ensuring, as far as possible, that:

The generated embryos will all be usable in our experiments (i.e. they will either carry the mutation we are interested in or will be suitable for use as an unaffected control).

Each litter has a chance of containing both experimental and control embryos (such that comparisons can be made within litters, minimising the variability between experimental measurements).

Experimental and control embryos are produced in roughly equal numbers so that there is no excess of one over the other.

In addition, whenever we are using only part of an embryo (e.g. only the heart, or only the brain), we will endeavour to cryo-preserve material from other tissues and organs such that it can be used in our future studies or shared with other interested researchers.

Where the safety of substances we want to administer to mice is not fully known, or it is not known whether the substance will affect the taste of diet or water, we will use pilot studies to establish, amongst other parameters, the minimum number of animals that should be included in a full study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are interested in human genetic conditions that cause developmental syndromes. To study these conditions, we need to examine the effect of the mutations that cause these conditions in whole embryos, so that we can observe the process of development and account for the interactions between different organ systems. Any animal model for these diseases must share genetic pathways for heart and brain development with humans, such that findings can be translated for medical benefit. It must also be possible to introduce mutations into the DNA of the animal model to mimic the effects of mutations identified in human patients. Mice are the most appropriate species for this project because they are the least sentient species that satisfies these two requirements.

Introducing potentially harmful mutations into mice could cause them pain, suffering, distress or lasting harm. To avoid such effects, we use "conditional" mice, in which harmful mutations are not found in parents and only become active in offspring when two unaffected adults are bred. These conditional models confine harmful effects to embryos at early stages of their development, where they have less capacity to experience pain, suffering and distress.

In some cases, we need to activate the harmful mutation at a specific time point during mouse development, which requires us to administer a substance to pregnant mice that converts the mutation to its harmful form. We administer substances using whichever method causes the least pain, suffering or distress to the pregnant mouse whilst still delivering our scientific aims. Usually, this means the substance is delivered through food, drinking water or a feeding tube.

Why can’t you use animals that are less sentient?

Animals less sentient than mouse embryos cannot be used because they do not have brains similar enough to humans for results to be medically actionable.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

When we administer substances to mice, we will monitor the animals daily to note any change in appearance or behaviour that might indicate a harmful event, and animals will be humanely killed if they exceed any of the specified end points. When substances are administered by injections under the skin on consecutive days, we will vary the site of injection to reduce the risk of inflammation. If there is any indication of reaction, advice will

be sought from the vet on an appropriate treatment. For example, if the skin is red at the site of an injection an anti-inflammatory cream may be applied. If the animal's condition does not improve after 24 hours, the animal in question will be humanely killed.

Pregnant mice will be handled using tunnels wherever possible and will be housed either in pairs or with a non-pregnant companion animal to reduce stress. Cages containing pregnant mice will be supplemented with a dome to provide additional safe enrichment.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will primarily use the resources provided by the NC3Rs (including the Experimental Design Assistant and the ARRIVE guidelines), Norecopa and the members of the Federation of European Laboratory Animal Science Associations. I will also periodically seek out any newly published guidance from the establishment Named Information Officer and Named Animal Care and Welfare Officer.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Everyone involved in the project will attend internal and external meetings and conferences focused on the 3Rs during the project. We will subscribe to the NC3Rs mailing list to ensure we are kept up to date about new events and developments. I will also consult with the regional NC3Rs manager wherever relevant.

18. Understanding Speciation and Adaptation in Cichlid Fishes

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

biodiversity, pollution, evolution, behaviour

Animal types	Life stages
Haplochromis spp.	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to understand which genetic changes are important in affecting behaviours involved in reproduction.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Understanding the genetic basis of reproductive behaviour can help us understand how populations become reproductively isolated and eventually become different species. Understanding reproductive behaviour can also help us identify any changes in this behaviour that environmental pharmacological pollutions could have, so that we

understand the wider ecological consequences of behavioural changes caused by pollution.

What outputs do you think you will see at the end of this project?

Outputs at the end of this project will mostly be publications, including post graduate theses. These will all be publicly available in repositories, and will typically include associated blog posts or press releases to widen accessibility of the results.

Who or what will benefit from these outputs, and how?

In the short term, most beneficiaries will be within the academic community, but longer term, understanding how biodiversity evolves will have conservation benefits. Understanding how pollutants such as SSRIs affect animal behaviour will also have relevance to e.g. environment agency, and other NGOs. Genetic tests of the effects of vision mutations on behaviour may also allow a fuller understanding of the genetic pathways involved in vision, which may ultimately provide medical benefits for humans.

How will you look to maximise the outputs of this work?

Videos and code will be made available so that the work is as reproducible as possible. Results will be published on BioRxiv ahead of publication in a peer reviewed journal, to maximise availability of the work, and will be accompanied by press releases or blog posts, and publicised on twitter to maximise dissemination. Results will also be presented at scientific conferences.

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

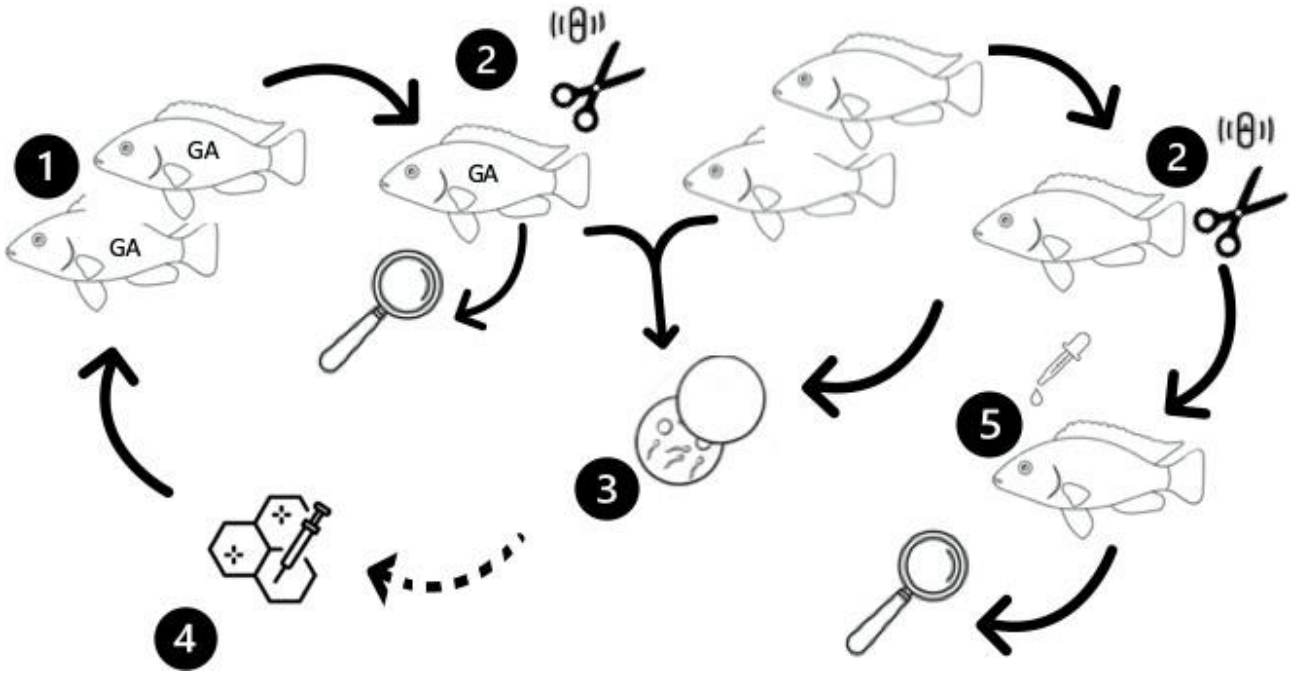
We are using these types of animals because they are a well studied model in evolutionary biology, and there is already a wealth of knowledge available upon which further work can be based. We plan to study the reproductive behaviour of these species, so we need to use adults for this. Where we genetically modify fish to better understand how specific changes to visual genes might lead to changes in behaviour, we use embryos, and raise these to adulthood so that we can observe reproductive behaviour.

Typically, what will be done to an animal used in your project?

Our project will involve inserting a small microchip in the abdominal cavity of the animal, similar to microchips used in identification for pets. This will enable us to identify individual animals and keep track of which other procedures they have undergone, so that they can be housed in stock tanks with multiple animals, which will reduce stress since they are naturally social animals. We will be able to record their movement across territories within their tanks. We will also take a small tissue sample so that we can extract DNA and identify what genetic changes the animal has - this will be either a small piece of fin no more than 1mm, or a skin swab with a cotton bud. Some of our animals will have a drug treatment, before being observed, to see how the treatment affects their reproductive related behaviours. This treatment will be administered in their water and will continue for 2-4 weeks, after which time they will be returned to normal water conditions.

One component of our project involves genetically altering fish, and this will be carried out at the egg stage, within one hour after the egg is fertilised. The genetic changes we make will mirror changes we find naturally in other closely related species, so we don't expect the animals to suffer any adverse effects.

The protocols involved in this project allow us the create and maintain genetically altered fish, and to treat fish with pharmaceutical drugs. Once we have done this, we will monitor and test their behaviour. The behavioural experiments themselves are observations and therefore are not regulated procedures. For this reason they are not explicitly described in any protocol, although the generation and maintenance of fish to use in these observations is.



There are 5 protocols in this project, and fish may be used in multiple protocols. In the diagram above, numbers correspond to protocols. Fish bred in captivity may be fin clipped and PIT tagged (2), then treated with pharmaceutical compounds (5) before being observed in behavioural experiments.
CRISPR-cas9 will be used to generate genetically altered fish (4) which will be bred and maintained in the aquarium (1) and maybe fin clipped and PIT tagged (2) if they will then be observed in behavioural experiments. Any fish, genetically altered, PIT tagged, or not PIT tagged may be used for producing gametes (3). The dotted arrow indicates that although genetically altered fish may be used to produce gametes for maintaining stock, they won't be subject to further alteration. Genetically altered fish will not be treated with pharmaceutical compounds.

What are the expected impacts and/or adverse effects for the animals during your project?

We anticipate that any adverse effects are very mild, and short in duration (i.e. for a couple of seconds during fin clipping or microchip insertion). These will be carried out under anaesthetic, post-operative analgesia will be used after consultation with the NVS using the most appropriate analgesic type and route of administration for the procedure involved. Typically when we carry out these procedures, the animals return to normal behaviour very quickly and live a long life in our aquaria.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities for all the procedures and animals are mild.

What will happen to animals at the end of this project?

- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to understand how changes at the genetic level, can lead to changes in reproductive related behaviours that can lead to changes in mate preference, reproductive isolation and eventually speciation. It isn't possible to monitor adult behaviour, without using adult animals.

Which non-animal alternatives did you consider for use in this project?

This project could not be carried out with non-animal alternatives.

Why were they not suitable?

Non-animal alternatives are not available for the study of animal behaviour.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We will try to use the minimum number of animals possible, but need to ensure that we use enough animals to account for the variability within the population. Behaviour is notoriously variable, and so samples need to be big enough to take this into account (details and power calculations are provided later). When making genetically altered fish, a large number may be required because there will be a relatively large number of fails but many fish will need to be screened in order to test this.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used data from previous experiments (ours and others') to assess what typical numbers might be needed for these experiments. We also designed the experiments so that there is periodic unblinding and analysis, so it is possible to stop adding replicates once a significant change in behaviour is detected.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To reduce the number of animals kept under regulated procedures we will strive to maintain the smallest number necessary to carry out a particular experiment, e.g. for each mutation line, only F0 animals with confirmed germ line transmission will be mated (or alternatively, sperms of which will be used to fertilise eggs from wild type (WT) females) to breed into adult F1 fish. We will also endeavour to preserve lines that are not currently being used by sperm freezing thus only maintaining actively used animals in the facility. In addition, the use of homozygous mutant animals allows us to reduce the total number of animals to characterise a gene.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use haplochromine cichlids in these experiments, for mild procedures with no lasting harm. We will carry out the procedures under general anaesthetic, and using post-procedure analgesic to reduce suffering.

Why can’t you use animals that are less sentient?

We can't use animals at a more immature life stage because we need to understand the effects of SSRI and/or vision gene changes on reproductive related behaviours, in order to understand the impact for evolution, and this is not possible on juvenile fish. Teleost fish are the most suitable because they have complex social interactions and reproductive behaviours, but are less sentient than other possible choices e.g. mammals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will minimise welfare costs by using anaesthetic and analgesic, and regular monitoring post procedure, including water quality where relevant.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Peer reviewed scientific publications centred around fish welfare, specifically with respect to tagging and behaviour, and the use of analgesics.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Advances in the 3Rs are shared on our intranet, I follow relevant researchers who share their findings and publications on twitter, and attend animal behaviour conferences, including RSPCA/NC3Rs conferences.

19. Regeneration and Tumorigenesis of the Respiratory Tract

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Respiratory, Regeneration, Cancer development, Repair, Stem cells

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to determine the cells and molecules responsible for maintaining an optimal function of the respiratory tract (nose, throat, airways, lungs, and ear) in healthy individuals. Also, to study the processes and factors that drive respiratory diseases and tumour development.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The respiratory tract mediates the vital processes of supplying oxygen to the blood and removing carbon dioxide. When air enters, it is distributed throughout the lung lobes by a highly-branched system lined by a continuous epithelial cell layer. This layer differs in terms of its structure and cellular composition along the tract, and consequently in its reparative and regenerative capacity. The respiratory tract is susceptible to injury as well as to the effects of aging and a range of diseases such as asthma, fibrosis and cancer. The cells and the mechanisms that control lung stability and those that drive regeneration and tumour formation remain poorly understood, but will be crucial in developing treatments for these conditions. That is why this project is important to increase our understanding of the factors regulating the respiratory tract balance and how aberrations in these can drive lung diseases and cancer. In addition, it will allow us to improve and identify new therapeutic approaches to ameliorate patient outcomes.

What outputs do you think you will see at the end of this project?

We will increase our understanding of respiratory tract biology in health and disease and in the development of cancer. This will enable us to identify and/or develop new therapeutic agents/approaches that could be used to treat human respiratory diseases, including cancer. The results will be submitted to scientific journals for publications, and could form part of intellectual property in case a new molecule or mechanism is discovered.

Who or what will benefit from these outputs, and how?

Patients with lung cancer and lung diseases suffering from poor quality of life will benefit from these outputs in the longterm. Data generated in this project will be used to increase our understanding of the mechanisms driving lung pathogenesis and, on identification of novel therapeutic potential, as preclinical data for drug development or establishment of a clinical trial.

Outputs from this project will also benefit the scientific community in the shorter term as our work will be disseminated through presentations and publications and this could lead to even greater scientific advancements.

How will you look to maximise the outputs of this work?

We are members of multiple consortiums and collaborations with world-leading experts working on mouse models of lung diseases and intent to make use of these to optimise the approaches used and to share new techniques; we will continue these collaborations for the upcoming years. We will also disseminate knowledge through presentation and publication.

Species and numbers of animals expected to be used

- Mice: 9500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To best mimic the human condition, the mouse models we intend to use have been chosen because they are widely accepted to be the most appropriate and relevant models.

The life stage chosen will be different in each protocol. For example, all ages will be used in the breeding and maintenance of genetically altered mice whereas only juvenile and adult mice will be used in tissue and cell grafting for regeneration as we want to understand the process in adult patients. The life stage chosen will always be the best to mimic the human condition.

Typically, what will be done to an animal used in your project?

• Animals will be dosed with different agents – the most common routes of administration are: instillation through the nose and/or the throat, The most commonly used include: aspiration through the nose and/or the throat, administration of agents via oral gavage, intra-peritoneal and/or intra-venous injection, application of agents topically or subcutaneously, change in diet and/or drinking water. These procedures are quick and will not take more than 2 minutes per animal.

• In some instances, surgery may be carried out to implant the agents, for example, in the kidney capsule, under the skin or in other specific locations in the oral-respiratory tract. This procedure takes more time and is done under general anaesthesia for no longer than 30 minutes.

• In some instances, animals will be pre-treated or treated with agents to modulate gene expression, injury or repair among other things.

• Animals may be imaged using minimally invasive imaging modalities.

• Where necessary, tissues/organs may be retrieved under terminal general anaesthesia with or without perfusion with a solution.

• All other animals will be terminated using a Schedule 1 method, after which time tissues may be collected

They may also be irradiated to facilitate tissue engraftment and some mice will be injected intraperitoneally with BrdU/EdU prior to termination of the experiment. These options will not be part of a typical experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of animals will be used in models of lung disease, tumour development or airway tissue engineering. These animals may develop tumours but these are generally well-tolerated. Some animals may experience reduced mobility, reduced lung function and some short periods of respiratory distress, but are not expected to show prolonged signs of breathing difficulties. In addition, some animals used in this project will experience weight loss but this will be closely monitored. A small proportion of animals will undergo a surgical procedure but these procedures are expected to pose minimal risk to animal welfare. Furthermore, some animals used in this project will be immune- compromised but these animals will be carefully looked after to minimise chances of infection. Studies will be designed to ensure only the minimum number of animals required are used. Throughout studies, animals will be regularly monitored; if any animal causes concern, action will be immediately taken to alleviate this and if this is not possible the animal will be humanely euthanised.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity will range from mild to moderate. Around 32% of mice will be under the mild severity category, and 68% in the moderate category, based on data from previous years.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The respiratory tract is extremely complex, with interactions between various cell types and different processes. This cannot be adequately mimicked using in vitro studies and therefore the most appropriate approach to mimic this is using a whole animal.

Which non-animal alternatives did you consider for use in this project?

In vitro, ex vivo and in silico experiments were considered. We intend to use these experiments to establish whether in vivo experiments are necessary.

Why were they not suitable?

Although non-animal alternatives will be used to establish whether in vivo experiments are necessary, the diversity of cell types and the complexity of the interactions cannot be mimicked using these techniques. The respiratory tract needs to be studied in a whole animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on previous experience and experimental plans.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We plan our studies so that only the minimum number of animals required is used and made use of the NC3R's Experimental Design Assistant.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will also make use of minimally invasive imaging techniques to enable us to monitor changes in a process longitudinally in a single animal, which will eliminate the need to use more animals at different time points. Wherever possible we will make use of alternative methods to genetically modify mice rather than by doing this through mouse crosses, which will reduce the number of animals generated unnecessarily. Wherever possible inbred strains of animals and littermate controls for genetically modified strains will be used to minimise the effects of genetic variation, which should reduce the number of animals required in an experiment. In addition, wherever possible we will use both male and female animals, which will reduce the number of genetically modified mice that are generated but not used in further protocols.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse models of lung diseases we intend to use have been chosen because they are widely accepted to be the most appropriate and relevant to the human condition they mimic. We have extensive experience in each of the chosen models, which allows us to reduce the number of animals required, to limit the invasive procedures carried out and to limit the discomfort experienced by the animals.

Why can’t you use animals that are less sentient?

Since we are interested in studying the respiratory tract, we need to use mammals, and the mouse models are well established and are the gold-standards in the field as they are the closest mimics of the human condition. Not only that, but the mouse respiratory system has been extensively studied and there are many reagents available that wouldn't be available for other animals. This project needs to be conducted on live animals to be able to study the mechanisms and interactions ongoing between the different cell types over time, and to allow us to study interventions.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Throughout this programme of work we will continue to monitor our own practices and the literature and the NC3R website to look for ways to refine our procedures; this could include monitoring the animals more closely and more frequently, providing additional nutrition and hydration to aid recovery after a procedure, changing pain management and giving antibiotics when needed. This makes administration of substances via this route significantly quicker and causes less distress to the animal. Environmental enhancement will be used to minimise stress (for example, soft bedding and wet mash where necessary). For chemically induced tumour induction models, in which animals are regularly treated for prolonged periods, animals are handled by the researcher prior to commencement of the experiment to allow the animals to acclimatise to the researcher and to minimise distress during the course of the experiment. In addition, in these experiments where chemicals are applied to the back of the mouse, a restrainer may be used to minimise the time required to apply the chemical, which minimises distress.

Wherever possible we will incorporate these improvements into our protocols to further refine, reduce and replace the use of animals. We will continue to use non- invasive imaging to monitor animals, which should enable us to identify earlier end points and to reduce the number of animals required.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

ARRIVE and PREPARE guidelines from NC3Rs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continue to monitor the literature and check the NC3R website regularly to be up-to-date with advances in the regulations. These changes will be incorporated into our protocols wherever possible. We will have regular discussions with the NVS and NACWO and will attend training offered whenever possible.

20. Investigating Disorders of Glucose and Energy Homeostasis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Metabolism, Energy Homeostasis, Diabetes, Obesity, Inflammation

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project will investigate the impact of altered energy homeostasis and how this affects organ systems and whole-body metabolism. By examining the effects of energy dysregulation on organ function and integrity, we hope to identify the underlying mechanisms that may lead to disorders associated with impaired energy homeostasis, such as diabetes and obesity

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The incidence of metabolic disorders such as diabetes and obesity are increasing exponentially. With an increasingly aged population, the incidence of complications associated with such conditions will become a substantial financial and societal burden. Improving our understanding of the mechanisms underlying these complications may improve our treatment strategies and minimise the long-term consequences.

What outputs do you think you will see at the end of this project?

The immediate outputs of this project will be scientific publications in high quality, peer-reviewed, high impact journals appropriate to the field of research, communication to relevant national and international scientific conferences and press releases disseminating the research findings

Who or what will benefit from these outputs, and how?

The findings of this research will, in the short term, improve our understanding of factors that may contribute to the development, progression and complications associated with metabolic disorders. For example, suppose a gene has been identified in a genome-wide association study (GWAS) relating to disturbances in glucose regulation. In that case, a GA model may be generated to look at the mechanism that may contribute to the manifestation of these conditions. In recent years the role of inflammation in the development of metabolic disorders such as insulin resistance, obesity and Type 2 diabetes is becoming more apparent. Indeed, there is debate whether inflammation is the cause or consequence of many disturbances in metabolism. Using in vivo models, we will explore the physiological (cardiovascular, renal, pancreatic), hormonal, inflammatory and cognitive consequences of changes in energy homeostasis. For example, the role of the GSK3/Nrf2 axis in beta cell decline in Type 2 diabetes will be investigated in a Diabetes UK funded project grant. Using GA modified mice, the constitutive levels of Nrf2 and GSK3 (alpha and beta) will be modulated to determine the potential role in the development of Type 2 diabetes. This work will generate mechanistic data to test the hypothesis that the GSK3-Nrf2 axis plays a crucial role in beta cell biology and identify therapeutic opportunities to prevent beta-cell damage, hopefully reducing the need for insulin therapy in type 2 diabetes.

In addition, the peripheral and cerebrovascular consequences of glucose and insulin dysregulation will be assessed in transgenic (e.g., Db/db.) and diet-induced obese (DIO) models of obesity and diabetes. This will provide mechanistic data to explore the links between diabetes and obesity with co- morbidities such as cardiovascular disease, cerebrovascular disease and Alzheimer’s disease (AD).

These findings will be disseminated to the broader community through interaction with our clinical collaborators, published in peer-reviewed journals, and presented at local, national, and international meetings. In the long term, the findings of these studies will be translated into the clinic. By working closely with our clinical colleagues, we hope to ensure that our animal models recapitulate the human condition as much as possible.

How will you look to maximise the outputs of this work?

The output of this work will be published in peer-reviewed journals and presented at National and International conferences. We will continue collaborating with fellow experts in metabolic disorders and other collaborators within the clinical research space. A paper is prepared to describe techniques that we have optimised (e.g., blood sampling from the tail vein without restraint) to disseminate this knowledge to the broader scientific community. The publication of negative data is crucial to minimising unnecessary experiments being performed, and this will take place via platforms such as BMC Research Notes.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use adult rodents (mouse) in this project as they are widely used in this field of research and share a similar physiological response to altered metabolism as make humans, i.e., weight gain and insulin resistance during nutrient and calorie excess. The use of transgenic models enables us to examine the impact of individual genes on metabolic disease progression or response energy deprivation/excess. The complications associated with metabolic dysfunction usually occur over several years in humans, and therefore, we will study adult rodents in this project

Typically, what will be done to an animal used in your project?

The animals used in this project will be studied throughout sustained energy imbalance (typically high- calorie feeding). Transgenic models aimed at disrupting key systems/signalling pathways involved in energy homeostasis will be utilised for in-depth analysis of the roles of these pathways in maintaining energy homeostasis and for investigating the physiological impacts of disruption of these systems in an in vivo model. We will then assess the effects of altered energy homeostasis on aspects of metabolic function and glucose handling in models of altered energy-sensing (transgenic,

pharmacological or diet-induced obese (DIO)). These experiments will typically last 2-3 months.

What are the expected impacts and/or adverse effects for the animals during your project?

We anticipate that animals will gain excess body weight (primarily fat mass) due to feeding of high- calorie diet (dietary-induced obesity – DIO) and begin to display symptoms of impaired glucose handling and insulin resistance following prolonged periods of positive energy balance. Symptoms may include polyurea (increased urination) and reduced physical activity. Animal husbandry will be adjusted to maintain a comfortable housing environment for these animals (remove and replace wet bedding) and body condition scores will be utilised to monitor animal welfare. So that we are able to "fix" the glucose levels in the body (referred to as "clamping" the glucose levels at a set concentration)animals may need to have permanent vascular catheters inserted into blood vessels in the neck under general anaesthesia. All animals undergoing surgical procedures will be given appropriate analgesia to minimise pain. Weight loss is likely to occur in the first two days following surgery, but support measures such as soft food will be provided to minimise weight loss. Guidance will be sought from the Named Veterinary Surgeon (NVS) and Named Care and Animals Welfare Officer (NACWO) should any animal display signs of abnormal behaviour or any unexpected change in physical appearance.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Adverse effects depend on the studies being performed. They mainly relate to surgery or the development of diabetes and obesity (approximately 50% of our studies), very much as we see in humans. Animals developing diabetes and/ or undergoing surgery are expected to reach the moderate severity category (approximately 20%). Genetically altered mice are not usually expected to display any clinical signs (subthreshold or mild severity), although some may develop diabetes and therefore potentially reach a moderate severity level (approximately 10%). Overall severity is expected to be 40% moderate, 60% mild/subthreshold.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Metabolic disorders such as diabetes and obesity are complex conditions characterised by a chronic energy imbalance, elevated blood sugar (glucose) and fat (lipid) levels, fat mass gain and alterations in insulin sensitivity (Type 2 diabetes). Although we can model some aspects of these conditions in vitro (for example, the exposure of cultured cellular models of metabolic tissues to nutrient and fuel excess), this doesn't fully replicate the interaction between different cell types or tissues that have a fundamental role in the development of many of the co-morbidities associated with conditions of chronic energy imbalance and altered metabolism, such as diabetes or obesity. Replicating the complex and variable conditions that occur in a disease state such as diabetes is extremely difficult and limits the applicability of these in vitro models to disease.

Which non-animal alternatives did you consider for use in this project?

Where possible, we perform studies in cell systems first to ensure that a candidate gene or pathway is essential to the things we want to study. Subsequently, highly specialised techniques have been developed to measure the animals' response to challenges, such as high-calorie diets. This means that we can compare much smaller groups of animals. Techniques have been developed to study the animal while awake and conduct repeated tests in the same animal rather than using lots of groups.

Why were they not suitable?

Replicating the complex and variable conditions that occur in metabolic disease states such as diabetes in vitro is extremely difficult due to the complex interaction between cell types, hormonal secretion and organ systems (pancreas, brain, liver, muscle, adipose tissue), and this limits the applicability of these models to disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals required for this project has been calculated based on previous experience using similar models.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experimental design will align with ARRIVE guidelines and follow NC3R guidance using the Experimental Design Assistant (EDA). Studies will be kept as simple as possible to maximise the

information obtained from the minimum number of animals. Advice will be sought as necessary from statistical sources, locally or online, concerning the minimum number of experimental animals required to allow a sufficiently robust statistical analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use highly specialised techniques to measure the animal's response to challenges, such as a high-calorie diet. This means that we can compare much smaller groups of animals. Plans have been developed to study the animal while awake and conduct repeated tests in the same animal rather than using lots of groups.

The mouse models that we propose to use in this project are well-studied models used frequently in metabolic research, which helps compare results to other groups. Transgenic models generated and studied during this project will be developed on well-established genetic backgrounds (e.g., C57BL6) for accurate comparison with existing literature.

In all cases, tissues will be collected and archived for subsequent analysis by both our group and others working in energy homeostasis disorders.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will induce diabetes (or glucose dysregulation) in many of the animals in our studies. Where possible we do this non-invasively by using high-fat or it naturally due to genetic changes. In some cases, we have to use a chemical by injection that destroys the cells that produce insulin in the pancreas. We have refined our injection protocol to make the solution less irritant and we ensure that diabetes caused is as stable as possible, using special small implants under the skin to release low levels of insulin if needed.

We minimise welfare costs by using highly trained staff to conduct all our studies with many years of experience working with animals and using our techniques. New staff are

rigorously trained. All studies are carried out following recommended guidelines under the guidance of the local veterinary team and in a facility with highly trained staff

Why can't you use animals that are less sentient?

Metabolic conditions are complex, and complications associated with these conditions develop over time. We cannot use non-mammalian models to study diabetes as this cannot occur other than in mammals. It is also not possible to use only animals under terminal anaesthesia as it alters the hormonal and biochemical response to fluctuations in fuels and therefore does not replicate the human condition.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

1. Before any experimental procedures, animals will be handled for a minimum of 1 week to acclimate the animal to the user and vice versa. Whilst on a study, animals will be weighed, and body condition assessed weekly by PIL and trained staff at the animal resource unit. Following surgery, post-operative care and pain management will be provided following advice from the NVS. Wherever possible, procedures will be performed in the animal's home cage following appropriate handling. Whenever possible this includes taking blood samples from the freely moving animals from the tail vein. This is a well-established procedure in the lab, and we have demonstrated it causes minimal stress to the animals.

2. The equipment used to assess whole-body energy metabolism (CLAMS) does so by indirect calorimetry, which allows for detailed measurement of energy expenditure. The CLAMS device allows for multiple, detailed non-invasive measures of energy expenditure in small groups of animals, reducing animal numbers required to measure each endpoint. The system assesses calorimetry (indirectly) via a closed system that takes measurements of energy expenditure at different temperatures by measuring changes in gases (02 and CO2) within each cage. Rodents have increased brown adipose tissue (BAT) compared with many other mammals and this means that temperature challenge is the best means of assessing energy balance in the body (energy homeostasis) dynamically.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Best practice guidance for experiments will be acquired from LASA, NC3R and other appropriate websites.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will regularly visit the NC3Rs website and others recommended by the local named information officer.

21. Immunity to Influenza Viruses in Pigs

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

influenza virus, pig, influenza vaccine, immune response, mucosal immunity

Animal types	Life stages
Pigs	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand protective immunity to influenza viruses in pigs. We will also determine how best to induce protective immunity by vaccines and evaluate novel therapeutics and monoclonal antibodies to prevent disease and virus transmission.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Globally approximately 1.5 billion pigs are produced annually for pork production, which accounts for more than one-fourth of total protein consumed worldwide. The demand for pork has led to intensification of production, with farms often housing thousands of animals leading to rapid pathogen transmission. Influenza virus infection in pigs is a major farming problem, causing morbidity, mortality and loss of productivity. Economic losses due to swine influenza (SI) are among the top three health challenges in the swine industry.

Furthermore, pigs are natural hosts for the same subtypes of influenza A viruses as humans and are integrally involved in virus evolution with frequent interspecies transmissions in both directions. The emergence of the 2009 pandemic H1N1 virus (H1N1pdm09) illustrated the importance of pigs in the evolution of zoonotic strains.

What outputs do you think you will see at the end of this project?

1) Characterisation of the specificity, magnitude, and duration of mucosal and systemic immunity to current and emerging influenza viruses.

2) Identification of early key events in the innate immune response that determine the outcome of infection and severity of disease.

3) Evaluation of the capacity of influenza vaccines and therapeutics to prevent clinical signs and limit virus transmission. The role of local and systemic immunity and how best to deliver vaccines and therapeutics to induce universal protection against different strains will be established. The part of the respiratory tract that should be targeted by vaccines or monoclonal antibodies for optimal protection will be identified.

4) Characterisation of the turnover of tissue resident memory cells (TRM) and their distribution in the respiratory tract.

The outputs of the project will be new knowledge about the nature of the disease caused by different influenza viruses and what are the protective mechanisms of immunity to influenza in pigs. In the short term we will also obtain information about the efficacy of novel influenza vaccines and therapeutics in preventing clinical signs of disease and limiting virus transmission. In the medium and long term these outputs will enable improvements to be made in the prevention of influenza virus infection in pigs and in humans. The outputs of the project will be disseminated primarily via scientific publications and conference presentations.

Who or what will benefit from these outputs, and how?

Pig farming industry: A key deliverable of this proposal is to determine how to make better and more efficient vaccines and control measures to limit the spread of disease. The beneficiary will be the pig farming industry. Both productivity and animal health and welfare will be improved, and secondary infections and severe disease reduced.

General public and human health: A more efficient immunisation strategy against influenza virus infection will also reduce transmission and the zoonotic threat posed by swine influenza viruses. Increased productivity in the pig industry will lower costs for the consumer.

As both pigs and humans are readily infected with influenza A viruses of similar subtype, the pig is a robust and appropriate model for investigating both swine and human disease. Like humans, pigs are outbred, and physiologically, anatomically, and immunologically similar to humans. The porcine lung also resembles the human in terms of its lung structure, physiology, morphology, and distribution of receptors bound by influenza viruses. Any knowledge gained from the pig model could be easily translated to humans for development of better immunisation strategies and treatments.

Food security and environment: Consequences of improved pathogen control include reduction in antibiotic treatment for secondary bacterial infections, the risk of contamination of the food chain and the environment, as well as the risk of developing antibiotic resistance.

Benefits to the commercial private sector: The knowledge gained in this project can be applied to other respiratory tract infections in pigs and also to other livestock species and humans. Our close relationships with industry will allow us to fast track the new immunisation strategies into field use. We will identify new correlates of protection that evaluate pulmonary as well as systemic immunity to respiratory tract infections and this will accelerate vaccine development in pigs, other livestock and humans.

Benefits to policy makers; International development: Development of control measures and better vaccines for animals and humans will have an enormous impact on health policy and quality of life throughout the world. Diagnostic and consultancy services are commissioned by the UK Department for Environment, Food and Rural Affairs (DEFRA) and equivalent organizations worldwide (EC DG- SANCO, the World Organisation for Animal Health and the Food and Agriculture Organisation), who will therefore also be primary beneficiaries.

How will you look to maximise the outputs of this work?

Knowledge generated by this project will be widely disseminated to the research community as soon as practicable through open-access peer-reviewed journals and presentations at national and international conferences, collaborative discussions and interactions with members of the scientific community. Outputs of this work will also be

distributed to other stakeholders and the general public through press releases and social media channels.

Species and numbers of animals expected to be used

- Pigs: 480

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We propose to use a mammalian host (i.e. pig) appropriate to the influenza viruses we are studying, such that key information, e.g. clinical/pathological data can be used by the farming industry, veterinarians, animal keepers and national veterinary authorities responsible for disease surveillance, disease outbreak management and policy formulation.

Pigs are a natural host for influenza viruses and their immune and respiratory systems are very similar to humans. Pigs are susceptible to some strains of human influenza viruses, especially H1N1pdm09, and human viruses or human-origin gene segments frequently adapt to transmit efficiently in pigs. The similarity of clinical disease and pathogenesis of influenza infection in the two species, make pigs an excellent animal model to evaluate novel vaccines. In our experience vaccines which are protective in mice and ferrets are not always protective in pigs, further emphasizing the difference between small and large animals.

The pig is therefore the most suitable animal to study immunity to influenza virus infection and to evaluate the effectiveness of vaccines and therapeutics. Weaned piglets will be used as they are most reproducibly infected with influenza viruses.

Typically, what will be done to an animal used in your project?

Typically, animals used in this project will be immunised by an injection of influenza vaccine candidate into the muscle or by administration into the nose or by aerosols into the lungs, for which the animals may be sedated. The immunisation typically will be conducted twice at an interval of 1 to 8 weeks.

Blood samples will be taken to characterise the immune responses. Immunised and unimmunised animals will typically be inoculated once with influenza virus into the nose. Nasal swabs will be taken daily in the first 7 days to evaluate the shedding of challenge virus. Blood samples will be taken weekly to characterise the immune responses. Animals will then be culled humanely to assess lung pathology and tissues will be collected to

assess virus load and for further analysis of immune responses. The typical duration of an experiment is 21-56 days.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals will experience mild and transient pain associated with a blood sample being taken, which includes restraint and insertion of a needle through the skin. Intranasal or aerosol administration of vaccines or therapeutics will be done using mucosal atomisation device or by aerosolisation, to reach the deep lung, using close fitting mask following sedation.

Our extensive experience using H1N1pdm09 and published data indicates that clinical signs of disease are consistently mild in pigs under experimental conditions, moderate being the highest severity ever observed, dependent on the virus strain used. The maximum severity recorded with H1N1pdm09strains is moderate, but in our extensive experience with these viruses the maximum severity is mild. The mild signs consist of intermittent coughing or a mild elevation in body temperature for 1 to 2 days. Rarely, animals infected with influenza viruses may develop moderate clinical sign consisting of laboured breathing, becoming lethargic or anorexic for 1 to 3 days. In this project, any animals which show these more severe signs will be killed if the duration reaches the endpoints in this licence.

In the case of unknown viruses (with unknown severity), pilot experiments will be performed, and challenge virus will be administered at times to ensure, wherever possible, that the peak phase of clinical impact falls within normal working hours, enabling regular observation. However there are provisions for out of hours checks. To allow severity to be monitored and minimised, clinical signs will be serially observed and recorded against systems-based clinical scoring sheets that include clearly defined end-points and intervention criteria. Pigs will be required to be anaesthetised transiently for some procedures and very rarely vomiting has been observed after anaesthesia. Pigs will be monitored throughout the procedure and recovery to avoid any adverse effects associated with the depression of body systems during anaesthesia and / or accidents during recovery

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

According to the cumulative literature and our extensive experience, 85% of animals are expected to show either no or mild clinical signs. Less than 15% are expected to show moderate clinical signs.

However, all animals will be clinically monitored both post-vaccination and post-infection. Assessments and interventions as appropriate will be performed at predefined frequencies in the experimental protocol, including euthanasia on welfare grounds if required.

What will happen to animals at the end of this project?

- Killed
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Due to the complex nature of the immune system, it is not currently possible to study immune responses to immunisation and infection and to determine whether they are protective without the use of animals. It is also impossible to study the lifespan of lymphocytes in vitro because we wish to understand the effect of the local lung environment on their survival and cell division. In vitro experiments will tell us how long lymphocyte can survive in tissue culture conditions but has no bearing on their survival in the lung environment. Therefore in vitro experiments cannot tell us how long immunity to influenza viruses could persist in the lung.

Which non-animal alternatives did you consider for use in this project?

Cell culture and molecular biology techniques will be used to characterise influenza virus in the laboratory before infecting pigs. We will use laboratory techniques to understand the responses of the pigs to infection with influenza viruses using blood samples and tissue samples taken post mortem.

Protection against respiratory diseases is carried out by white blood cells in the lung, called tissue resident memory cells (TRM). After establishing how best to induce TRM we shall perform a series of in vitro experiments to determine what factors influence their maintenance and survival.

We shall also use a primary porcine airway epithelial cell model and tracheal organ cultures to study virus replication, anti-viral therapeutics and vaccines for the control of influenza. We have established for the first time in vitro porcine tonsil, lymph node and spleen organ cultures to dissect antibody and T cell responses. The parallel use of in vitro models and in vivo studies, will validate the predictive value of these in vitro models.

Why were they not suitable?

In line with the objectives of the programme of work a complete biological system is required to study transmission and protective immune responses to influenza virus infection. Therefore, in vivo studies have to be performed to provide critical data and biological materials that correspond to the outcomes and responses to viral infections in the animal itself. Given the nature and localisation of the TRM it is not possible to use an in vitro system to study how these cells are induced following immunisation, nor is it possible to measure immune responses to or protective efficacy of a vaccine without the use of animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers to be used have been estimated using data previously collected from similar studies and in consultation with a statistician.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The experiments are designed to ensure the appropriate number of animals are used - numbers are selected that enable robust experimental design compatible with obtaining reliable and meaningful results. The advice of an experienced biostatistician from our establishment will be sought, as well as from other collaborators as appropriate. The animal studies are designed to maximise collection of biological materials/data from each study and enhance the development and use of in vitro and ex vivo methods where appropriate.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- Using inbred large white pigs (Babraham pigs) in some studies may enable us to obtain significant results with smaller groups of animals since these pigs are 85% identical as assessed by genome-wide SNP analysis and are matched for MHC type I and type II molecules.

- When investigating novel antigens, tissue culture analyses in the laboratory are conducted where possible to refine the number of candidate antigens for evaluation. This allows for a reduction in the number of in vivo studies required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animal experiments are designed with close consideration of the likely overall severity and the period of peak severity caused by administration of a defined and consistent dose of virus (for example between 1 x 106 to 5 x 106 pfu per animal for H1N1pmd09) based on previous experience and published information.

We have developed a purpose built mask for aerosol administration which allows maximum exposure in the minimum time.

Sample collection will be carried out within clearly defined limits specified in individual experimental protocols, and repeated sampling will be done at frequencies such that the method of sampling causes no more than momentary pain and suffering and no lasting harm.

Substances administered to animals by injection or intranasal inoculation will be made in the smallest volume commensurate with the aims of the procedure according to good practice guidelines

Because of the rapid air changes in the rooms in the high containment facilities at our establishment, in order to best mimic natural room air, a purpose built Perspex pen (246 cm x 246 cm x 140cm) high is used for the contact challenge which allows the animals to exhibit their natural behaviour.

The severity level after influenza infection is mild in most cases. However, some pigs may develop moderate signs of disease which will not exceed the specified humane endpoints. Symptomatic treatment as agreed with the veterinary surgeon will be provided to alleviate suffering whenever possible.

Pigs housed in the high containment facilities at our establishment are provided with various enrichment items, including a straw bed, hanging toys and a rotation of ground objects to interact with. We have developed an enrichment monitoring program to help define which enrichment items are useful, and how frequently they should be rotated etc.

Why can’t you use animals that are less sentient?

Pigs are natural hosts for influenza viruses and a source for novel zoonotic strains, which may cause epidemics or pandemics in humans. Mice cannot be infected with most strains

of the influenza virus and do not recapitulate signs of illness observed in pigs and humans; guinea pigs do not exhibit overt signs of illness; and ferrets may have different drug pharmacokinetics to pigs and humans. Only in a natural host such as pigs, is it possible to dissect the pathogenesis of disease and identify how to control the virus.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Pigs are intelligent animals and so enrichment is particularly important to create a stimulating environment. They are given straw beds which also enables them to express their species-specific behaviour of rooting and investigating. They are supplied with a variety of fruit, vegetables and toys which are frequently rotated.

Where possible, pigs are trained with positive reinforcement to reward desired behaviour e.g. cooperating with procedures which could include swabbing without restraint. This is a refinement in animal handling methods to improve animal welfare and the value of animals in research. Animals are also housed in pairs or groups to allow for normal social interaction.

Highly trained animal technicians will monitor these animals throughout the day, ensuring they are comfortable and to maximise their welfare status. We have 24/7 CCTV surveillance which can be used to monitor the animals' behaviour over time.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Adherence to the ARRIVE guidelines for reporting these studies, as well as reference to the FELASA guidelines for pig health monitoring to help ensure the most robust health assurance for animals used in this study. FELASA guidelines for administration of substances has been used to limit the maximum volumes for each of the routes.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will attend appropriate 3Rs conferences, read the relevant scientific literature including the veterinary literature on pain relief, and undertake regular project licence holder training and refresher courses. I will take advantage of news and information provided by the NTCO. I will also use other sources of information such as:

- The NC3Rs
- (AALAS) American Association for Laboratory Animals Science
- (FELASA) Federation of European Laboratory Animal Science Associations
- (ICLAS) International Council for Laboratory Animal Sciences

22. Regulation of the Mammalian Reproductive Axis

Project duration

5 years 0 months

Project purpose

- Basic research

Translational or applied research with one of the following aims:

  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Infertility, Reproduction

Animal types	Life stages
Mice	adult, juvenile, embryo, neonate, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to study how mammalian reproduction and fertility are regulated by the interplay between different genes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Globally, infertility affects around 5% of all couples so the number of individuals with fertility problems is enormous. In many cases, the underlying cause of the infertility is not known reflecting out incomplete understanding of the factors that can cause these problems. A number of infertility problems will be caused by genetic mutations and it is important to study these to try to identify ways in which the infertility can be treated.

What outputs do you think you will see at the end of this project?

The main output from this research will be a contribution to scientific knowledge including data sets and publications in peer reviewed journals and presentations at scientific meetings. Specifically, we will generate novel data and knowledge about the role of specific genes in mammalian fertility. For example, we will define the genes that control the activity of Kiss1 neurons and how these might change during puberty. We will also define the way in which neurons involved in fertility are regulated by hormones (eg sex steroids) or peptides in the brain (neuropeptides). We will also define the function of the Hiat1 gene in controling male fertility.

Who or what will benefit from these outputs, and how?

The immediate short term benefit of this research is that it will provide training to stakeholders that are involved with the project and contribute to our knowledge of the mammalian reproductive axis and how it is controlled. The project will contribute to the research training of undergraduates, PhD students and post-docs. In the longer term, the project will also provide a greater understanding of clinically relevant disorders such as precocious or delayed puberty and may allow the development of treatment regimes. The knowledge from this project might also be of benefit for the development of approaches to treat disorders in women (eg cyst formation in the ovary or post-menopausal hot flushes) or might allow the development of novel male contraceptives by phamaceutical companies.

How will you look to maximise the outputs of this work?

The outputs will be disseminated through peer reviewed publications, reporting findings at scientific meetings, on the departmental web site and via social media. Where possible, we will also try to report negative findings in peer reviewed publications and on my personal web page which is accessible to all.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Genetically modified mice are a powerful resource to understand the function of a gene in a whole animal system as the technology exists to allow the distruption of a specific gene and examine the consequences of this. This project is examining puberty and fertility so it is necessary to use mice at these stages (prepubescent and adult).

Typically, what will be done to an animal used in your project?

The majority of the mice (5000, 90% of total number) are part of the breeding colony to generate a smaller number of mice (750, 15%) for experiments. The genetically modified mice in the breeding colony will not usually have any procedures performed on them, unless a second earclip is required for genotyping, and will not have any detrimental effects. From the breeding colony it is estimated that 350 mice (7% of total number) will only have injections of substances, and a small number (400, 12.5% of total number) will have surgery with recovery.

Most protocols will only take a few weeks to complete although mice undergoing pharmacological treatments will be kept for longer to allow recovery after any surgical procedure. Within the pharmacological treatment protocol, 350 mice will have minor procedures (injection and/or blood sampling). 400 (12.5% of total number) mice will have one surgical procedure (ovary transplantation or gonadectomy or substance delivery into the brain - icv). Up to 50 mice (1% of total number) will have two surgical procedures (gonadectomy and icv delivery). The mice that have undergone surgery may be kept for 12 months to allow long term monitoring of repoductive parameters.

What are the expected impacts and/or adverse effects for the animals during your project?

Breeding of the GA mice is not expected to be associated with any detrimental effects or losses apart from those normally associated with maintaining a breeding colony.

Surgical procedures will be carried out using aseptic techniques. In the uncommon event of postoperative complications, animals will be killed unless, in the opinion of the NVS, such complications can be remedied promptly and successfully using no more than minor interventions. In case of wound dehiscence, an uninfected wound may be re-closed on one occasion within 48 hours of the initial surgery and the NVS informed.

Peri- and post-operative analgesia will be provided in accordance with a regime agreed in advance with the NVS Animals are expected to make a rapid and unremarkable recovery from anaesthesia. In the uncommon event that animals fail to do so, or exhibit signs of pain or distress or of significant ill health, they will be killed by a Schedule 1 method.

Unless otherwise specified, the administration of substances and withdrawal of bodily fluids will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm. We have

not observed any adverse effects from administration of hormones, which are normally given in the normal physiological range. Any animals showing any deviation from normal health or well-being will be killed.

A minority of the animals may experience weight loss within 15% (measured against their weight at the beginning of the protocol or compared to age matched controls, as appropriate), mild piloerection, intermittent and moderate hunching, and reduction in their activity within the cage. From the time point of the surgery when these symptoms may be expected to occur, animals will be closely observed every 4 hours during the day to ensure that the symptoms would not last for more than 12hours. If these signs persist for over 12 hours or begin to deteriorate the animals will be killed by Schedule 1 methods. This is not expected to occur in more than 1% of the animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild 5100 mice (92% of total number)
Moderate 450 mice (8% of total number)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are no suitable alternative systems that can recapitulate the complex interplay of the different tissues (brain/pituitary/gonads) of the reproductive axis or the hormonal feedback mechanisms involved in its regulation.

Which non-animal alternatives did you consider for use in this project?

The only non-animal alternatives that could be considered are using tissue explants or established cell lines. Established cell lines that could be used to model the physiology of Kiss1 neurons include the mHypoA-50 and mHypoA-55 lines and the KTaV-3 and KTaR-1 lines. While these lines show some responses found in Kiss1 neurons in the mouse such as a response to estrogen, which makes them suitable for some experiments, they cannot model the neuronal circuitry found in the intact brain.

Why were they not suitable?

Tissue explants are not suitable for this project because they cannot be maintained in culture for long periods, they will change as soon as they are removed from the animal, they do not recapitulate the reproductive axis and are not subject to the normal regulatory signals (eg fluctuating hormone levels) that occur in a whole animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of transgenic mice that will be used over the 5 year duration of this project is based on the number of mice that have been used each year on my previous PPL. Over the course of this licence, we will generate around 5,000 mice (using approximately 170 mice each year for experimentation). Of these, the majority (around 4,150) are used for breeding and these will have no detrimental effects caused by the genetic alteration. As we are studying mutations that affect fertility, the mutant mice are sterile so we have to breed from heterozygous pairs which means that many mice will be wild-type. A further 100 wild-type mice will be used each year for the generation of genetically modified mice and these will not have any surgical procedures performed on them as I reoutinely perform non-surgical embryo transfer. Surgical procedures (mainly removal of testes or ovaries) will be performed on around 400 mice with delivery of substances into the brain performed on around 150 of these.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When appropriate, the NC3R's Experimental Design Assistant (https://www.nc3rs.org.uk/our- portfolio/experimental-design-assistant-eda) and the NC3R's Breeding and Colony Management Resource (https://nc3rs.org.uk/our-portfolio/breeding-and-colony-management) will be used to plan an experiment and to define the minimum number of mice in each treatment group to achieve statistical power. We also have standard operating procedures (SOPs) in the laboratory to help ensure standardisation between different staff.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To minimise the number of surplus mice that are bred and not used in experiments, we routinely keep breeding pairs together for only a couple of months and then stop breeding until we require new offspring. We actively monitor and manage the number of breeding pairs so that we only have the number that is required to generate a cohort of offspring for a specific experimental plan. Pilot studies might be undertaken to get preliminary data on the variability of experimental measurements to allow us to perform Power calculation to determine the minimum number of mice required for statistical integrity. We use surplus wild-type mice from our breeding stocks for control groups rather than buying in additional mice. We also provide surplus mice for tissues to other groups.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Transgenic mice with disruptions of specific genes involved in fertility (Kiss1, Kiss1R, Hiat1) will be used in this project. Mice are an excellent model to study mammalian reproduction. The main physiological processes are conserved between mice and humans and the hormonal regulation of fertility in particular is almost identical between these species. In addition, the ability to generate transgenic mice, in which a single gene has been altered or removed, provides a very powerful tool for studying the role of a single gene in reproduction. They represent much better and more specific models to study brain function than models involving crude destruction of brain areas. The genetic approaches to generate these models are very well established in the mouse and are efficient enought to allow transgenic mouse generation while keeping the mouse numbers low. In addition, the short gestation period of the mouse allows us to study aspects of reproductive function relatively quickly.

The transgenic modifications carried by the mice do not cause any detrimental effects or pain - the vast majority of the mice will only be killed and used for tissues.

Why can’t you use animals that are less sentient?

It is not possible to use a less sentient model organism (eg nematode worms, Caenorhabditis elegans or fruit flies, Drosophila melanogaster) to study the mammalian reproductive axis. They do not have the appropriate tissues that are found in the mouse or the same hormone systems.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have undertaken a number of refinements in our animal welfare procedures and will strive to continue to do this. For example:

We have developed an ultra-sensitive method for measuring a hormone in the blood, which works with very small volumes of blood (5 ul) so that we can reduce the amount of blood taken from the tail vein.

We will retain existing ear clip tissue taken for identification purposes and use this for identifying the genetic make up of the mice [and thus eliminate the need to re-earclip for genotyping alone.

Transfer of embryos into recipient female mice to generate genetically modified mice will normally be performed using an NSET (non-surgical embryo transfer) device, which is a less invasive method than surgical transfer of embryos.

Where possible, we will use genetically sterile mutant male mice instead of vasectomised males.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the NORECOPA online resource (https://norecopa.no/prepare) for guidance on quality assurance and animal management and as an aide memoire for the topics that should be considered when planning experiments.

We will refer to the various publications on the LASA web site (https://www.lasa.co.uk/current_publications) for information and specific guidance about many aspects of animal research.

We follow the ARRIVE guidelines for publishing our work.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

My establishment has a knowledge distribution system (mainly via e-mail and a Biomedical Support web site) to keep all PPL holders informed about advances in the 3Rs. In addition, I frequently check the NC3R's website for advances and subscribe to their Newsletter. I will also have regular discussions with the Named Persons and animal technicians to review current approaches and whether there are any new 3R opportunities. I have also subscribed to the Norwegian NORECOPA Facebook page as this organization provides useful information and resources about the 3Rs. I will also check the LASA web site (https://www.lasa.co.uk/) for information and I have set up an e-toc alert for the ATLA (Alternatives to Laboratory Animals) publication.

23. Xenopus Embryonic Development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Embryogenesis, Cell Differentiation, Heart Development, Wnt signalling, Cancer

Animal types	Life stages
Xenopus laevis	adult, embryo, neonate, juvenile
Xenopus tropicalis	adult, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims at improving our understanding of embryonic development, that is, how a properly formed body emerges after the fertilisation of an egg by sperm. This project focusses on very fundamental processes, processes that are conserved in backboned animals, which therefore allows us to study these processes in frog embryos as they develop into tadpoles, yet does provide us with valuable information about processes also important for human embryonic development. Studying such fundamental processes is much easier in frogs (compared to human or mammalian development) since fertilisation and subsequent embryonic development of frog tadpoles occur outside the maternal organism and can therefore be observed and experimentally studied without carrying out experiments on the adult organism. Particular fundamental processes that we focus on include Wnt signalling and heart muscle development.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

As embryonic cells differentiate (that is: different cells take on different functions and associated shapes, e.g. some cells become neurons while others form muscle) they communicate with each other in the embryo using biochemical cell-to-cell signalling mechanisms, such as particularly Wnt signalling mechanisms. Wnt signalling is not only fundamentally important in frog and human embryonic development, it is also involved in human disease, such as cancer; thus, studying Wnt signalling mechanisms in frog embryos (including how it interacts with other regulatory mechanisms) can provide valuable fundamental scientific understanding that is also potentially relevant for human embryonic development and for congenital birth defects as well as human disease (such as cancer). Because of our research focus on Wnt signalling and on heart development, our findings are likely to be important for understanding Wnt signalling in human cancer and about congenital heart defects; and possibly also for recovery from heart attacks (myocardial infarction).

What outputs do you think you will see at the end of this project?

The fundamental nature of our research probably means that most outputs will be in the form of new information that will be disseminated at scientific meetings and in scientific publications.

Who or what will benefit from these outputs, and how?

The fundamental nature of our research probably means that in the shorter term the immediate beneficiaries will be other researchers and scientific and medical research using other experimental systems. They will benefit from attending the same scientific meetings as us (for instance on Wnt signalling or on Heart development and cardiovascular biology and medicine) and by reading the scientific literature we publish or reviews of our findings by others. However, our fundamental research is very much aimed at providing the kind of new information that will in the longer term benefit human health and health provision. Due to the nature of our major research questions, this is most likely going to be in the area of cancer and heart defects. Beneficiaries could of course also include biotechnology and health industry translating aspects of our findings into products for the health provision or further research in the same specific area or more widely. The end benefits in terms of changes in clinical practice etc. are likely going to be much longer term.

How will you look to maximise the outputs of this work?

We will maximise outputs of our research by collaboration with others, including particularly collaboration within our own research group with researchers using findings

from the accessible frog experimental system to confirm in human and mammalian systems, including in cultured human stem cell differentiation and organoid systems.

Species and numbers of animals expected to be used

- Xenopus laevis: Over five years: 35 adult females (based on reuse), 65 adult males, 250 genetically altered tadpoles.
- Xenopus tropicalis: Over five years: 35 adult females (based on reuse), 65 adult males, 750 genetically altered tadpoles.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The specific research addressing our fundamental scientific questions can be carried out on fertilised frog embryos developing towards tadpoles before they become sentient (before they develop the ability for feeding). These early stages of embryonic development are not regulated since they precede the free feeding stage when it is thought the tadpole or the froglet and then frog developing from it can experience pain or suffering.

However, as part of this research we intend to produce genetically altered animals, while again aiming to study any experimental effects during early embryogenesis, any effects on late-stage tadpoles or the froglets and then adult frogs cannot be ruled out and needs to be carefully monitored.

Typically, what will be done to an animal used in your project?

In order to study frog embryonic development, we need to harvest sperm (from adult male frogs) and eggs (from adult frog females). In order to encourage egg production (and typically also for sperm production) the adult frogs are injected with fertility-promoting hormones (gonadotropin). In order to encourage egg laying the females are typically handled in a particular way so as to mimic what the male would normally do during amplexus. Males are typically culled to dissect and isolate testes used in in-vitro fertilisation.

What are the expected impacts and/or adverse effects for the animals during your project?

These procedures have been highly refined for almost a century for Xenopus laevis and have been improved in the last few decades for Xenopus tropicalis. Normally these procedures therefore only mildly impact the animals.

However, handling (during the hormone injection or during encouraged egg laying) can occasionally cause an adverse effect on the delicate skin of these aquatic amphibians, which can develop (skin sloughing, occasionally as visible abrasion) within 12 hours and normally improves within 24 hours and must essentially be cleared within 72 hours.

However, occasionally in first-manipulation Xenopus tropicalis (and very seldomly in Xenopus laevis and manipulation-experienced Xenopus tropicalis, particularly when not used for several months) following fertility hormone injection, substantive numbers of eggs are not properly laid and can then subsequently develop an egg-bound phenotype (rotting eggs in the abdomen).

Any adverse effects of genetically altered frogs have not actually ever been observed but represents a theoretical concern depending on the particular genetic alteration.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity is mild

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The interaction of genes in the embryo is complex because of the three-dimensional structure of the embryo itself. In order to understand these complicated interactions of genes they have to be ultimately confirmed in this three-dimensional landscape of the embryo.

Some aspects of the function of these genes can be studied in isolated tissues (see below), which allows a reduction of whole-animal experiments, however, discoveries from artificially isolated tissues will still need to be verified in the natural three-dimensional setting of the embryo, before they can be relied upon.

Which non-animal alternatives did you consider for use in this project?

Some in our research group are using stem cells in culture to study suitable particular aspects of the relevant biology in this project (such as in-culture cell differentiation of pluripotent embryonic stem cells into functional heart muscle cells).

Why were they not suitable?

Some aspects of the relevant biology can indeed be studied in cell culture, and we use it already to study relevant mechanisms in a more directly medically-relevant mammalian and even human cell culture system. However, often only aspects of the normal biological and genetic mechanisms operate in cultured cells and therefore discoveries from cell culture systems need where possible be verified in the natural three-dimensional setting of the embryo, before they can be relied upon. Cultured cells and tissues lack the three-dimensional complexity of normal tissues and organs and therefore also lack important signalling interactions between different cell lineages that would occur in normal tissues and which are relevant for gene regulation. In heart tissue for example this leads particularly to differentiation of only immature heart muscle compared to differentiation of more normal mature heart tissue in whole embryo systems. However, we are currently exploring ways of improving cultured systems, such as three-dimensional organoid systems to improve them. But currently, a whole embryo system particularly the experimentally accessible Xenopus model system is still indispensable.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimation are based on the proposed experiments requiring about 20 weeks' worth of animal experiments per species per year, the interval suitable for re-use of adult females (longer for X. laevis than X. tropicalis), how long isolated sperm is usable (longer for X. laevis than X. tropicalis), the estimated rate tadpoles can successfully metamorphose to adults (based on some experience of our own with wild-type and advice received from Xenopus resource facilities in UK, US, and Japan).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Since the experimental design concerns the number of fertilised embryos, but the number of animals influences the likelihood of getting sufficient embryos to carry out the planned experiments, the experimental design does not in a linear way determine the number of animals used. In practice, many more scientific experiments can be carried out on an

experimental day when a given number of adult frogs are used to produce a lot of embryos for experiments, while the same number of adult frogs on a different day will on occasion only produce few eggs of sufficient quality for scientific experiments. Thus, in practice optimising the quality and reliability of harvested fertilised eggs is therefore more important for the regulated procedures than the number of adult frogs (quantity) used.

However, the number of embryos used will be carefully considered in the experimental design so that the use of animal resources leads to statistically supported results: In the Xenopus field for each experimental condition we ensure we have a sample size of at least 30 embryos, where we analyse the embryos individually (e.g. whole-mount RNA in situ hybridisation or immunocytochemistry). We use at least 15 embryos or 20-30 explants for pooled analysis (e.g. quantitative RT-PCR, Western analysis, RNA-seq, ChIPseq, more if more material is required) to produce continuous values (e.g. ratio of expression to control). Before publication we also repeat the experiment on three different days (involving different males and females to produce sperm and eggs, respectively) and therefore analyse at least three completely independent biological replicates.

Any power calculations of sample size numbers required for a particular experiment is however preliminary until the size of the observed change (effect size) between control and experimental samples is known. More experimental embryos will therefore need to be analysed if a more subtle effect is found and needs to be substantiated. The significance of actually observed effects will be assessed by Student’s t-test (continuous scoring) and Chi-squared test (discrete scoring) or by ANOVA (for more complex multivariate data sets). Professional statisticians at the licensed establishment regularly provide advice on statistical tests.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Optimising the re-use interval for the different species is important, as well as, identifying and recording carefully which animals are more reliable to produce products (mostly eggs from females) so that their re-use is a better use of animal than use of a naive animal. This will ultimately reduce the number of individual animals used, but also since it will be more reliable, this will reduce the number of regulated procedures needed to produce the products (mainly embryos) for scientific experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We propose to use adult Xenopus laevis and Xenopus tropicalis to produce eggs and sperm for scientific research.

The protocol for Xenopus laevis females has been developed for approximately a century and is very refined and should normally only at most cause mild levels of severity. The protocol for Xenopus tropicalis females has been developed for decades, it is also very refined; however, we find (and the wider tropicalis research community finds that first-use of Xenopus tropicalis females can experience severity levels exceeding mild.

It is of course difficult to be sure, but at the moment it appears the difference is inherent to the different species, rather than a lack of sufficient refinement of the protocol for Xenopus tropicalis. The use of Xenopus tropicalis is however justified since the diploid genome (like humans and most other vertebrates) is required for genetic and genomic studies, including those proposed here; which would be much more difficult if not impossible with the allotetrapoid genome of Xenopus laevis.

We have therefore introduced more detailed and more frequent monitoring for signs of ill health during recovery (at least twice per day) in the two days following first-use in regulated procedures for Xenopus females. We will continue to do so throughout this programme for Xenopus tropicalis, and will regularly review whether it is also necessary to continue to do so for Xenopus laevis.

The hormone injections needed for encouraging Xenopus females to mature oocytes and then lay eggs is more reliable when animals are not feed in the two days before the first hormone injection; and the subsequent egg collection (by any method) is more efficient if laid eggs are not intermingled with faeces. Xenopus are cold-blooded (poikilotherm) and therefore do not need to take in food as often as warm-blooded animals (endotherm, such as birds and mammals). However, food will not be withheld for longer than nine days for Xenopus laevis or five days for Xenopus tropicalis. The difference is due to the colder temperature (leading therefore to slower metabolism) at which Xenopus laevis is kept relative to Xenopus tropicalis.

Why can’t you use animals that are less sentient?

The actual scientific experiments are carried out on non-sentient embryos.

Sentient adult Xenopus frogs are required for harvesting eggs and sperm to producing embryos for those scientific experiments. No other stages are available that are less sentient yet can produce eggs or sperm to producing embryos for those scientific experiments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are now successfully refining an increased monitoring regime for particularly naive first-use Xenopus tropicalis females following the procedure. Information from the UK national Xenopus community also suggests that a more frequent re-use of Xenopus tropicalis females (i.e. more frequent than every three months) is actually beneficial to how the individual females react to the procedure.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Xenbase (model system international community web site) e.g.:

<http://www.xenbase.org/common/jsp/showWiki.jsp?Protocols>

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

There is a close network of experts in the UK national (and the international) Xenopus community; particularly for Xenopus tropicalis this provides the most effective and specific source of expert information. We are also regularly briefed by the NIO and by communications by the HO, our current funders and other funding agencies.

24. Bioelectronic Medicines II

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Neuromodulation, Physiology, Autonomic nervous system, Peripheral nerve

Animal types	Life stages
Sheep	adult, juvenile
Pigs	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The purpose of this body of work is to provide further evidence that the process of neuromodulation, alteration of an organ’s function by targeted delivery of an electrical stimulation to a specific neurological site or nerve, can be an efficacious alternative, or addition, to pharmacotherapy to treat broad ranging diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Modulating the nervous system (neuromodulation) for the treatment of locomotor, visual, auditory, and genitourinary dysfunction is a well characterised procedure in pre-clinical modelling of disease. This typically involves the implantation of electrodes encapsulated in devices, coupled with a battery power supply, to electrically excite the nervous system, to restore function that was previously lost through trauma or disease. It can also be used to record activity of the nervous system, to detect instability in the system and disease states, as well as block signals that have become pathological. This field of study is now referred to as neuroprosthetics and is now showing great promise as a potential first line therapy in the clinic, for intractable loss of function in patients after injury to the nervous system or with limb amputation.

It is now clear that similar neuromodulation techniques could be applied for the treatment of diseases associated with organs/systems such as diabetes, hypertension, rheumatoid arthritis, and inflammatory bowel disease, medically refractory epilepsy, or myocardial infarction among many others, through modulation of the autonomic nervous system. The autonomic nervous system is a component of the peripheral nervous system that regulates bodily functions, and which may be positively affected with modulation. For example, application of stimulatory devices that modulate the vagus nerve, an autonomic nerve that controls many organ functions, has shown great effect at treating animal models of disease.

The purpose of this work is to provide further evidence that neuromodulation to control organ function can be a viable alternative, or addition, to drug based therapy to treat a broad range of diseases, such as those mentioned above.

We will first ascertain whether novel targeted modulation of the autonomic nervous system can be accomplished in specific organ systems. This will be undertaken in anaesthetised animals. We shall establish an anatomical and physiological understanding, including determining the optimal interrogation point for the nerve to organ. Secondly, in a recovery setting, we will assess the surgical application of the device, the effect on the physiology over multiple applications of the therapy, and the surgical recovery profile .

We need to use large animal models, specifically sheep and pigs, because the size, anatomy, and physiology of these animals is translationally applicable to humans. Our devices are designed for human-scale anatomy, and the farm pig is the most suitable model for this when targeting nerves and organs of the viscera. Pigs are also well characterised with respect to metabolic and inflammatory pathways, and closely resemble

the human physiology. Physiology and anatomy of the sheep will be relevant in cases of implants to the neck, and so these models will be justified and used in those scenarios.

What outputs do you think you will see at the end of this project?

The project aims to test the feasibility of new treatments for patients that suffer progressive diseases (including inflammatory and metabolic disorders), through modulation of the autonomic nervous system. Areas of application include immune-inflammatory, and metabolic disorders. Examples of these disorders include rheumatoid arthritis and type 1 and 2 diabetes, with around 14 and 415 million sufferers worldwide respectively. A common problem with these patients is that they become refractory to pharmacotherapy and the therapies themselves often result in debilitating side effects. The long- term benefit of this work is to provide these patients with a novel and more focussed (patient specific) treatment approach through modulation of the autonomic nervous system. Most of these diseases are life-long and thus substantial benefits accrued by this treatment (over traditional pharmacotherapy and molecular based medicines) are in reducing cumulative suffering, enhancing quality of life and reducing the cost of life-long treatment.

Overall, our efforts in advancing this new treatment modality of neuromodulation will be focused on providing a greater resolution on the mechanism of action of this treatment in the short term. Medium term benefits are to determine efficacious parameters and safety profiles for the surgical implantation of a device and the levels of electrical stimulation of the nerve. This early-stage data will allow predictions to be made on the efficacious levels (strength and duration) of neuromodulation, as well as to support refinement of the device design that could be brought to the clinical market, while refining the surgical approach for implantation. Additionally, immune-modulating and anti-inflammatory therapies through modulation of the autonomic nervous system might be effective treatments for some or all forms of epilepsy. An immediate scientific output resulting from work modulating the autonomic nervous system before and during seizures is to allow further understanding of the pathophysiology of Sudden Unexpected Death in Epilepsy (SUDEP). The longer-term aim for patients is to aid in decreasing seizure frequency and to reduce the risk of premature death in epilepsy patients.

Heart attacks (Myocardial infarctions (MI)) are another area of disease which could benefit from this type of treatment. Current treatments to reperfuse the heart can lead to further rebound injury (reperfusion injury) to the muscle due to inflammation and despite the vast number of treatments for MI there is still a large unmet need and no specific treatment directly affecting reperfusion injury. The goal, and significant benefit to patients, is to develop a targeted therapy to reduce reperfusion injuries and prevent further damage to the heart muscle by the use of autonomic nerve modulation.

The outputs from this study will be publishable data which will be disseminated through peer review journals and meetings (we intend to do this after each set of experiments if appropriate).

Additionally, the output may be data for regulatory studies and patentable products which can be commercialised and offered clinically to patients (once regulated studies have been completed).

Who or what will benefit from these outputs, and how?

Studies in large animals will provide stronger evidence for the translation of these new therapies into humans, providing an anatomically and physiologically relevant platform to validate the implantable device, in both acute and recovery settings. Typically, industry takes about 10 years to successfully take a device from animal to man. Under our previous project licence we have reduced time by 3 years.

In the first instance the data generated from studies will aid the researchers in the selection and characterisation of new surgical implantations which will lead to their further development (e.g. in clinical trials) and could potentially lead to new therapies for immune-inflammatory, and metabolic disorders being introduced to the market.

Long term, these products have the potential to significantly enhance the quality of life for people suffering these chronic diseases or potentially cure them. This will benefit the whole society via reduction in absenteeism from work or school and reduction in demand on health services.

How will you look to maximise the outputs of this work?

Where confidentiality agreements allow, our commercial clients will publish the information via peer- reviewed scientific journals and conference presentations in addition to patent applications.

One of the key goals of our academic clients and collaborators will be the dissemination of results in seminars, conferences, and peer-reviewed articles with open access, in order to promote the general advancement of the fields studied. Negative findings may be published to avoid duplication of work by other groups.

Species and numbers of animals expected to be used

- Sheep: 75
- Pigs: 650

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult sheep and pigs will be used because they have a nervous system that is sufficiently anatomically and functionally similar to that of humans and thus enable the final safety and efficacy testing of these devices. These two species are required because the specific target nerves and surrounding anatomy are slightly different between species, and we require the anatomy, physiology and surgical access to be as close to humans as possible in different scenarios. For example, sheep are anatomically & physiologically better suited for upper airway/vagal work while pigs are a better fit for studies involving the abdomen.

The anatomy of the sheep and pig nervous system is well defined, enabling robust application of surgical methods and implantation studies.

Typically, what will be done to an animal used in your project?

After arrival, all animals will be allowed to become acclimatised to the new environment.

Some animals may have blood samples taken and / or receive a pharmacological agent, for example, to modulate the immune system.

The majority of animals will be terminally anesthetised and used in a non-recovery study. Prior to surgery animals may be fasted (but no withdrawal of water) for up to 24 hours. During non-recovery surgery, animals may have implantable devices to allow neuromodulation and nerve recordings, some may undergo imaging (such as CT), samples (such as blood and organ biopsies) may be taken. Just prior to termination, under the anaesthesia, from some animals there may be collection of samples and / or biological materials, to avoid euthanising further animals for this purpose.

A proportion will be anesthetised, undergo a surgical procedure and allowed to recover. Prior to surgery animals may be fasted (but no withdrawal of water) for up to 24 hours. The surgical procedure may involve implantation of a neuromodulation device, imaging, placement of a permanent intravascular catheter. Samples may also be collected. After recovery the animals undergo a series of therapeutic subclinical neuromodulation (levels of nerve stimulation which have been determined in the terminal studies to be efficacious to produce modulation of the target organ but shown to not have off- target side effects). Imaging, such as radiology, may be performed in conscious animals. Pro- inflammatory substances may be given and blood samples may be collected. This may be repeated on a number of occasions over a 12-month period. These animals may undergo a second recovery and / or terminal surgical procedure and if recovered the subclinical neuromodulation and/or pro- inflammatory substances dosing will be repeated. Finally, the animal may undergo a non-recovery terminal procedure or be killed.

What are the expected impacts and/or adverse effects for the animals during your project?

The larger proportion of animals used in these studies will not experience any adverse effects as the studies will be conducted under terminal anaesthesia.

With protocols and procedures that involve animals having recovery anaesthesia, some adverse effects for the animals are expected. Animals may experience discomfort and pain following surgery.

We will monitor this and provide the appropriate level of pain relief and post-operative care. Other effects include weight loss and changes to behaviour and general overall condition. We anticipate these adverse effects being transient and with good monitoring, welfare management, and nutritional supplements these effects can be alleviated. We would expect the animals to show full recovery within 7 days of surgery. If this is not the case the animals will be humanely killed.

With our specialist large animal vets, we have established clearly defined humane endpoints in all our models that minimize discomfort and pain to the animals yet allow us to address our scientific questions.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For the majority of animals, the studies will be conducted under terminal anaesthesia and the severity level will be non-recovery. However, as stated above, in some studies the animals will be recovered following surgery and may experience some adverse effects, but these would only cause the animal a moderate level of distress.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Computational modelling testing has been done without using animals to give confidence that nerve stimulation may treat disease. The science cannot be advanced further without using animals. Only a whole-body system biology approach will give conclusive evidence and understanding that manipulation of the nervous system can be an effective treatment of disease.

A computer model does not yet exist to test nerve stimulation as a treatment of disease.

Which non-animal alternatives did you consider for use in this project?

Computational modelling is relatively recent in the field of neuromodulation, it has already been successfully utilised in several neural engineering applications. Prior to starting in vivo work, we conducted simulation studies representing approximated nerve behaviour. We used multiple neurostimulation scenarios to determine stimulation current-, charge- and charge-density requirements for nerve recruitment in porcine and human splenic neurovascular bundle (SNVB). We then validated our modelling data by measuring electrophysiological parameters in freshly explanted human splenic neurovascular bundle obtained from organ transplant donors after ethical approval and informed consent. We stimulated the explanted SNVB in an ex vivo electrophysiology preparation using a bipolar cuff electrode and recorded the stimulation-evoked compound action potentials (eCAPs) using downstream hook electrodes. These approaches have enabled the determination of clinically relevant stimulation parameters for implantable device requirements for in vivo work and ultimately to go into humans.

Why were they not suitable?

Computational modelling may one day replace animal modelling in this context, however there is still considerable gap in scientific knowledge around all aspects of the nervous system including anatomy and physiology. Therefore, there comes a point when the computer modelling cannot completely answer the questions and a full intact working nervous system is required. At this point the use of animals for these studies is the only option to test therapeutic efficacy of bioelectronic medicines

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These figures have been calculated on the numbers used under the previous project licence and future plans. Up to the end of 2021 approximately 400 pigs were used under the previous PPL. Predictions for pigs for 2022 mean this number is likely to reach 500 by the end of the current PPL. The number of sheep used was lower than predicted for the previous licence. Because of this the number of sheep for this licence has been reduced.

With the ongoing projects planned for the next 5 years, (expected to increase) and additional projects intended to be started, the predicted number of animals included on this licence is a realistic estimation of the total usage over the 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To ensure we use minimal number of animals required to obtain meaningful and relevant data, we have extensively consulted available literature, attended experimental design and statistical courses, discussed with statisticians and NC3R staff and information provided by the NC3R. All requestors for work under a service contract will be required to justify the number of animals required and if appropriate show how they came to this number. The ARRIVE 2 guidelines have been referred to and followed where appropriate.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Definitive studies with sufficient animal numbers to obtain statistically significant results will be performed only after pilot studies have been performed to develop optimal methodology and assess feasibility and outcome measures. Electrophysiology methods are robust and repeatable, with minimal variation between animals. This provides clear results that give us confidence in making decisions even from small sample sizes.

In many cases, the numbers of animals required will be reduced by longitudinal measurement of responses, e.g. by serial blood sampling, multiple stimulations of nerve preps. Tissue samples at the end of studies can be collected to obtain the maximum information from a study. Also, samples taken may be used to inform other studies and provide extra data which will help advance the project or field as a whole.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Pigs and sheep will be used for all experiments because they are the most appropriate species to determine efficacy and safety with respect to the devices tested. Their neuroanatomy and physiology are very similar to that in humans.

We will work with manufacturers and academic experts to ensure a continued refinement approach is adopted for all implantable devices, electrodes and leads. We will work toward fully implantable devices as advancement to external wires and head caps.

Recovery studies will only be performed once surgical technique, application of neuromodulation and treatment parameters have been defined as much as possible using terminally anaesthetised animals.

All methods used for recovery animals will be refined to minimise any pain; these include appropriate provision of analgesia, use of local anaesthetics where possible for blood sampling and close monitoring of animals by large animal veterinarians and advanced trained animal technicians to recognise any adverse effects. All animals will be trained and habituated to the environment, staff and handling techniques prior to study to minimise stress.

Systemic inflammation models are well-characterised and used experimentally in clinical and non- clinical studies to determine the efficacy of medical treatments for immune / inflammatory diseases. Further development of chronic low-level inflammatory models will allow investigation of a range of diseases and the potential benefits of Bioelectronic medicines and therapies in these diseases.

Why can't you use animals that are less sentient?

We need to use large animal models, specifically sheep and pigs, because the size, anatomy, and physiology of these animals is translationally applicable to humans. Our devices are designed for human scale anatomy, and the pig is the most suitable model for this when targeting nerves and organs of the viscera. Pigs are also well characterised with respect to metabolic and inflammatory pathways, and closely resemble the human physiology here. Physiology and anatomy of the sheep will be relevant in cases of implants to the neck, and so these models will be used in those scenarios.

A large part of the project will be performed using terminally anaesthetised animals to define the treatment parameters of neuromodulation. Recovery animals will only be used when these parameters have been defined.

In addition, studies using rats and mice are undertaken as part of this research and help inform these large animal studies; this work is covered by a separate Home Office project licence. Studies involving pigs and sheep are one of the final steps within the project when similarity to human nerves is essential.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To minimise discomfort/harm to the animals, most studies are non-recovery in terminally anaesthetised animals with defined humane endpoints.

All animals will receive appropriate peri-operative care in terms of anaesthesia and pain management both during and after the procedure.

Our in-house large animal vets' expertise further enhances animal welfare by providing close collaboration with dedicated animal care staff and ready access to highly skilled advice. Specific recovery plans have been designed to ensure the best recovery of any animal post-procedure and involve high levels of monitoring.

All animals are habituated to the environment and all recovery animals are trained prior to use for all handling procedures, such as use of a restraining crate.

Least invasive route of substance administration, appropriate needle gauge and local anaesthesia will be used where possible. Negative control groups (baseline groups) will be minimised whenever statistically feasible.

All individual study plans are reviewed including consideration of justification and implementation of refinement and reduction as part of the local protocol review process.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the NC3Rs guidelines on the "Responsibility in the use of animals in bioscience research" and consult all the relevant references listed therein, e.g. NC3Rs Blood sampling resource.

(Reference: NC3Rs/BBSRC/Defra/MRC/NERC/Royal Society/Wellcome Trust (2019) Responsibility in the use of animals in bioscience research: expectations of the major research councils and charitable funding bodies. London: NC3Rs.)

For substance administration the LASA substance administration guidelines will be consulted (Reference: Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider. J Am Assoc Lab Anim Sci. 2011 Sep; 50(5): 600–613.)

Animals will continually be monitored for signs of pain and distress, especially post-operative. Post- operative care will be given by specialist large animal technicians and Vets.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continuously monitor publications and the NC3Rs website for new and alternative models that could be implemented as part of this project. In addition, articles on advances in the 3Rs are regularly published on the internal Users News Forum and other relevant information is circulated by AWERB. Whenever possible we will implement these refinements into our studies.

25. Treatment of Short and Long Term Outcomes of Viral Lung Infection

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Lung, Virus, Bacteria, Immunity, Pathology

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Lung viral infections cause devastation globally every year as evidenced by the last coronavirus pandemic. We will discover new treatments for lung viral infections and the ongoing complications that arise.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Understanding the role of the immune system in lung viral infection is key to the development of therapies that alleviate severe disease before vaccines are available. Some of our discoveries are being tested in patients already, but not all people respond in the same way. Our aim is to define a suite of treatments that will cover the majority of those severely affected.

What outputs do you think you will see at the end of this project?

Outputs will include:

1. A greater understanding of how the immune system is affected by changes in structure during infection.

2. Publications disseminating our findings to global scientists.

3. Development of concepts with the pharmaceutical industry

4. As demonstrated in our last project license, transfer and testing of our discoveries in relevant patient groups using new products.

5. Dissemination of understanding to the general public via articles in, for example, the public journal The Conversation and interaction with Public and Patient groups

Who or what will benefit from these outputs, and how?

Mid- to long-term the beneficiaries will be the pharmaceutical industry (new targets for development), and particularly the patients who are already benefitting from our discoveries. The health care system will also benefit. For example, our recent trials of new therapeutic reduced the length of hospital stay, reduced the number of patients requiring admittance to the intensive care unit and decreased long term outcomes. This reduces the stress on the NHS and the financial burden of lung infection.

How will you look to maximise the outputs of this work?

We routinely use a Patient and Public involvement group from the British Society of Immunology to capture facets of disease that most concern the patients. This disseminates new knowledge.

My team hold regular REDDIT sessions, which is an online forum for anyone to ask questions on the topic we propose. The last one was on COVID-19 where in two hours we had over 1000 questions to answer. This platform also drives interactions between all participants, even when the session has finished.

I regularly contribute opinion pieces to "The Conversation" that has an online reach to thousands of subscribers. In the last year my pieces have made the top number of interactions in my establishment.

I regularly respond to National and International media interview requests regarding viral infections. I have been inundated during COVID-19.

I have a policy to publish all research, not just those that produce a positive outcome. Negative data is equally as important to prevent others going down the wrong path. Negative data will be published as per other findings in peer reviewed journals and on the Wellcome Trust open access site. The data is also placed onto the group webpages and raw data files shared

Species and numbers of animals expected to be used

- Mice: 6000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The reason we use standard laboratory mouse strains is because over many decades they have proven to be readily infected with respiratory pathogens via the natural route by intranasal or aerosol administration. As in humans the pathogen replicates in cells lining the airways. Even viral strains that are not mouse pathogens induce a pneumonitis that, similar to humans, is reduced by early treatment with antivirals. The virus divides with similar speed to that in man. Symptoms of disease, like that in man, are dependent on the virus strain and the amount of virus administered. The route of infection, site of replication, replication kinetics and the specific cells recruited to combat that infection are identical to that in man.

We use adult mice, typically over 8 weeks of age as they yield enough cells to perform many experiments per mouse which reduces the need for separate experiments to address different questions. We have tried to replace animal use by using cell lines, but unfortunately a complex system is required to understand long term consequences of lung infection.

Typically, what will be done to an animal used in your project?

In a typical experiment mice will be housed initially in groups of 5. To induce a viral lung infection mice are first anaethetised by inhalation of anaesthetic. Virus solution is applied to the nostrills, which is then inhaled by natural breathing. Mice recover within a few minutes. It takes a maximum of 10 days to recover from the infection. Occasionally, during this 10 day period mice are given treatments by injection. At time points after infection, mice are killed by excess anaesthetic and multiple organs taken to assess pathology and the effect of any interventions. In a few experiments, mice will be left to recover from the viral infection for up to 6 month (typically 3 weeks) and then given a bacterial infection to

see the impact of previous viral infection to general immunity to infection. Other than infection, we may also breed mice that are not available commercially.

What are the expected impacts and/or adverse effects for the animals during your project?

About 50 % of the mice will not have an infection and so will not experience any adverse events. In those mice given a single infection with viruses respiratory inflammation and weight loss may occur. Weight loss appears gradually from day 3, peaks on day 6-10 and full recovery to their original weight occurs by day 10 after infection. Approximately 50 % of animals are likely to experience weight loss. This is because they have been given an intranasal infection that causes inflammation with influenza like symptoms. The remaining 50 % of mice have mild or no symptoms from the infection per se as the infection does not cause weight loss or they have been treated with control substances or therapeutics that reduce inflammation. At the peak of illness mice may show a hunched posture and reduced mobility, but in the main, all mice recover. Transient pain may occur on injection, though in most instances they are anaesthetised when this procedure occurs. Some weight loss may occur in mice given bacteria alone.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

This project works to a maximum severity of Moderate. This status is determined by a number of factors including degree of weight loss (maximum of 20 %), mobility, posture and overall condition. Mice are weighed daily and observed. In some cases mice have neared maximal weight loss but are still alert and in good condition. Instead of culling the mouse, we monitor the trajectory of weight loss (the steepness), breathing and general condition, twice daily. This prevents unneccesary termination of the experiment which would require another experiment to be set up. 70 % of mice in our protocols only experience up to 15 % weight loss

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Viral infection causes complex problems, and much of the disabling effects exist long term. The life- threatening illness to infection is caused by the interaction of many body systems. The illness also depends on cross-talk between immune cells and structural components of the lung. During our last project grant we discovered that immune cells are influenced by alterations in tissue structure and have taken this idea into patients and the treatment of those patients. Our discoveries will reduce the need for animal experiments in the future as we focus more on patients. However, we still need to discover more treatment options as one size does not fit all.

Cells cultured in the laboratory do not take into account blood and air flow or the impact of the nervous system. Nor do they show drug distribution. Replacements would be ideal in the current economic climate as animal experimentation is costly and time consuming.

Which non-animal alternatives did you consider for use in this project?

I strongly support the use of non-animal alternatives and have considered the following:

1. Matrigel systems that contain 2-3 different cell types ,
2. Organ on a chip,
3. Tissue slices,
4. Human lung resection, and
5. Human transplant lungs

Why were they not suitable?

Alternatives 1 and 2 still do not represent the lung microenvironment . Nor to they replicate the long term build up of complications over time. Furthermore they do not show how a virus infection leads to bacterial complications in the future, which is a critical medical issue in many patients with underlying lung conditions. Cellular systems also do not allow analysis of repair and the impact of altered repair.

In 3 and 4 we routinely use material collected as part of lung resections in patients with cancer, focussing on "normal" lung 4 cm from the cancer mass. This tissue has effectively reduced the range of experiments performed in mice and we will continue to use this approach. However, the tissue is highly criticised for its suitability and we cannot address bacterial super-infections or the repair process using it.

In an attempt to make the lung resection more representative we initiated discussion with lung transplant teams at our local hospital. Despite long negotiations, transplant material not used in patients is still unavailable. This tissue would still not replicae bacterial super-infections or the repair process well.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

There is an extensive literature using in vivo models of respiratory infectious disease and the applicant has greater than 20 years of experience. The overall number of animals is considerably reduced from the previous license due to the progress made. The estimated numbers in each of the three protocols is based on using 4-5 mice per group, which we have found to provide strong statistical power that reduces the necessity for multiple repeats. Initial therapeutic testing used 5 mice per group which is reduced to 4 mice per group depending on the reproducibility of the effect in the first study. We will not rely on historical precedent however to determine sample sizes as this may lead to serious over- or under-estimation of animals required. Data collected in preliminary experiments will be used to compute the sample size needed in follow up studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The estimated number of animals to be used has reduced dramatically from the last licence since I am no longer supervising the early career academics that used my license for their experiments. The experimental design used for the remaining protocols has reduced animal use in the following ways:

1) Sharing: With correct timing and consultation, we have determined that multiple scientists, asking different questions can use the same mice. Mouse number are based on 2-3 scientists sharing mouse use. In practice we have reached seven sharing.

2) Reducing repeats: It is possible to repeat observations whilst also progressing the research to the next question. Direct repeats are discouraged.

3) Confidence in reproducibility: Over the years of experience the reproducibility of the model has become established. In this license we can therefore be sure of a statistical significance with 4-5 mice per group.

4) Use of NC3R resources: There are online resources available to test null hypotheses (where you take an educated guess that something is true) or the alternative hypothesis (the opposite of the null hypothesis). These resources tell you how many comparisons need to be made within experiments and between experiments. Relevant

resources are also present for randomisation, blinding and allocation. These resources have been used to assess the number of animals in proposed experiments

All personnel using this licence will make optimal use of the experimental design assistant (EDA)and the scientific implications of poor design choice.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Animal reduction will be facilitated by group members sharing tissue and employing rigorous experiment design developed by our statisticians to calculate the least number of mice needed to address our questions. Where an experimental type is used more than once we will assess performance over time to ensure that it is continuing to perform well. The laboratory has archived specimens that can be used by subsequent researchers. Unfortunately storage processes can affect our results and so these archived tissues have limited use. As proof of principle however, they are adequate.

At our establishment we have instigated a community of researchers for sharing. As an example, we have a germ free mouse colony and when some are used in an experiment the details (age, gender, number per group and time points) are put onto a Teams platform. Members are therefore aware of the experiment and indicate whether they wish to collect additional tissues. This has been incredibly successful and in some experiment lung, gut, skin, bone marrow, kidney, blood and brain have been sampled from the same group of mice, reducing animal use by 7 fold compared to users working individually.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will only use mouse models that have been refined over the years to minimise animal distress and replicate the human scenario better. Procedures involve administration of virus and bacteria by inhalation and injection of immune modulators to reduce disease severity.

Minimisation of animal suffering critically depends on close monitoring of mouse condition (body weight, mobility, the quality of mouse fur, presence of cyanosis and whether they cling onto the cage lid when weighed). Staff perform this inspection daily, or twice daily if

concerned. See scoring criteria below. We try to avoid intraperitoneal injections where possible, using intradermal or subcutaneous where the alternative exists. We will refer to the Joint Working Group on Refinement. The latter, we feel is less intrusive than intraperitoneal. Where appropriate procedures are performed under inhalational anaesthetic that allows recovery within a few minutes. This prevents mice from losing body temperature whilst immobile.

Body weight	No weight loss	0	
	0-5% weight loss	1	
	>5 -10% weight loss	2	
	>10-15% weight loss	3	all experimental animals will be placed on mash
	>15-19% weight loss	4	If weight loss continues for greater than 72 hours, mouse must be sacrificed
	>19% weight loss		Cull Immediate action must be taken.
Ear positioning	Normal	0	
	Slightly retreated	1	
	Retreated completely back	2	
Coat and skin condition	No piloerection	0	
	Slight Piloerection	1	
	Marked piloerection	2	
Social interaction	Normal	0	
	Isolated or withdrawn from group	1	
Posture	Normal	0	
	Abnormal posture (hunched)	1	
Posture and mobility	Normal	0	
	Hypoactive	1	
	Lack or reluctance to move when stimulated	2	
Inactive	Cull		Immediate action must be taken.

Why can't you use animals that are less sentient?

Standard laboratory mouse strains are readily infected with respiratory pathogens via the natural route of the nose. As in humans the pathogen replicates in exactly the same lung cells. Even viral strains that do not usually appear in mice induce an inflammation that, similar to humans, is reduced by early treatment with antiviral medicines such as amantidine and ribavirin. Symptoms of disease, like that in man, are dependent on the influenza strain and the dose of virus administered. The route of infection, site of pathogen growth and the immune cell response are identical to that in man. Unfortunately, none of this can be replicated in less sentient species. Mice of a younger age are technically

difficult to infect and fail to represent the majority of infections that occur in adults and the elderly. Furthermore, early age responses to infection are different to adults.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

An academic at the establishment and the named veterinary surgeon have developed a severity score monitoring system (see above). To minimise animal suffering and recognise when humane end points have been met, they have created an objective scoring system that will be applied to every experimental animal. To generate this scoring system, we have selected welfare indicators that are relevant to this scientific study, are practical to carry out and do not disturb animals during assessment.

Expected adverse effects

- Some animals (5-10%) may experience temporary (less than 24 hours) symptoms (erect fur, respiratory symptoms) following intranasal exposure. Mice are given general anesthetic and there is some risk of pain or death (less than 1%) during the procedure.

- During this time-period animal welfare will be monitored daily

- Weight loss remains the most accurate predictor of mortality, and so body weight will be monitored daily.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow:

Joint Working Group on Refinement

The government animal testing and research: guidance for the regulated community (https://www.gov.uk/guidance/research-and-testing-using-animals)

The NC3Rs webpage https://www.nc3rs.org.uk/

Standard Operating Procedures developed with the University facility and the named veterinary surgeon.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All personnel associated with this project license will routinely interact with the NC3Rs online resource (https://www.nc3rs.org.uk/). Updates from the website will be a standing item on the group agenda where advances will be discussed.

26. Cutaneous Immune Responses in Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:

Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

immunity, skin, therapy, inflammation, infection

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to identify and characterise pathways and mechanisms within, and associated with, the skin that regulate inflammation and immunity during homeostasis and disease. As well as contributing to an understanding of disease pathogenesis, we aim to translate our findings to changes in clinical practice.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The skin frequently represents the first point of contact with pathogens and allergens, yet we still know relatively little of the role of the skin barrier and skin immune system in reacting to such challenges.

The immunopathology of inflammatory skin disorders, including psoriasis and eczema (atopic dermatitis (AD)), combines barrier dysfunction and immune dysregulation. This is exemplified by studies that identified the gene encoding structural barrier protein filaggrin as a key genetic determinant of AD, and the efficacy of currently available drugs that targets proinflammatory immune responses in AD clinical trials to date.

The skin is more than a superficial line of defence and has recently been suggested to have wide reaching systemic influence. Psoriasis is linked to multiple comorbidities including psoriatic arthritis, cardiovascular disease, atherosclerosis, obesity, diabetes, inflammatory bowel disease and psychological disorders. Classically, AD forms the first step of the “atopic march”, the progressive development of non-cutaneous allergic disease, including allergic asthma, rhinitis and food allergy. More recently AD has also been linked to cardiovascular disease. As such work to understand and target skin immune responses is important with wide ranging implications for health and disease.

Commensal bacteria are a part of a healthy skin barrier, which can be dysregulated by disease, as observed with the increased skin density of Staphylococcus aureus in at least 70% of AD cases. Skin colonising bacteria associate with skin infections, inflammatory disorders, more serious systemic infections and non-healing wounds. As such there is a need to understand the interaction of the cutaneous immune system in homeostasis and inflammatory disease with commensal and pathogenic microorganisms and the dysregulation of commensal status, which is key to the development of treatments for skin disorders.

The aim of this project is to investigate fundamental regulation of cutaneous immune responses to further understand the mechanisms of skin inflammation and related diseases, and aid optimisation of approaches to drug and vaccine delivery to this important tissue.

What outputs do you think you will see at the end of this project?

Outputs will include publications describing mechanisms of skin inflammation, pathways of antigen recognition in the skin and draining lymph nodes, and intervention regimen for pathological skin inflammation conditions. Outputs will also include patent applications and candidate compounds with therapeutic application in skin inflammation diseases.

Who or what will benefit from these outputs, and how?

The immediate core benefit of the work will be that we will increase our fundamental understanding of the molecular and cellular pathways that regulate skin infection and

immunity, with particular focus on inflammatory disorders and allergy. With this knowledge we can work towards better prevention of disease (through vaccination, prophylaxis and good practice), improvement in diagnosis at an early stage to increase the benefit of treatment and/or reduce transmission, and development of new therapeutics. These are long-term goals, beyond the 5 year duration of this licence, but the objectives of this project are to provide some of the necessary information, for us and the scientific community, to make this possible.

Our research to date has enabled us to identify new therapeutic targets and develop potential drugs for the treatment of skin allergy and inflammatory disorders, and continuing with these studies under this licence will enable us to support work to take these drugs forward towards clinical trials. In addition, we will continue to pursue the identification of new treatment strategies, for example by using disease models to identify or validate molecules and pathways that can be targeted therapeutically.

The characterisation of recently developed transgenic animals within this programme of work will also be valuable to other scientists aiming to develop therapeutics to these and related diseases by facilitating more rapid progress in their investigations.

Novel findings, delineating pathways in skin inflammation and novel reagents used for modulating these pathways will be published within the 5 year duration of this licence. Patent applications and assessment of products that will be developed for preclinical testing and clinical use may extend beyond the time frame of this licence (beyond 5 years).

Where widely applicable new treatments with public interest will be developed, a wider publication audience will be sought through our funders and local communications advisors.

How will you look to maximise the outputs of this work?

Dissemination of new findings will initially be through lectures, conferences and subsequently in scientific publications, which will be available through an Open Access route. My colleagues and I present our work at national and international conferences where we can discuss work in-progress before publication. This can generate new ideas and lead to collaborations. Indeed, we have a number of collaborations world-wide, with research scientists and clinicians, in which we provide expertise or reagents, and allows us to draw on the experience of colleagues and take advantage of the most advance technology that we can access.

Where possible unsuccessful approaches will be embedded in publication and discussed in meetings.

Species and numbers of animals expected to be used

- Mice: 15,500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are currently the "lowest sentience" of mammalian species in which these experiments can be performed robustly and reproducibly. The murine immune and haematopoietic systems closely resemble the human systems and many murine models of human disease have been developed and characterised. The mouse also benefits from well-established and robust technologies for transgenic and gene-targeted genetic alteration. In addition, there is a large body of published scientific evidence that exists to be built upon and referenced to.

The diseases under investigation are normally associated with juvenile to adulthood therefore, this is the choice of life stage in our studies. However, typically mice are 6-8 weeks at the time of experiments.

The diseases that are modelled in mice often differ in some aspects from the equivalent diseases in human (e.g. models of AD are more acute than chronic, as in human disease). Nevertheless, when examining aspects of the diseases such as the response of specific cell types to treatment, there are sufficient similarities to make conclusions that are transferable to human biology and disease.

Typically, what will be done to an animal used in your project?

Our experiments in mice will mimic, in an experimentally controlled fashion, challenges to the cutaneous immune system, including infection, autoimmune disease, allergy and inflammation. We will administer substances by a variety of routes, choosing the most suitable and least invasive route possible. Our models involve creating inflammation or infection in the skin that mimics such conditions as psoriasis, contact/irritant dermatitis, and atopic dermatitis (allergic eczema). These will be generated by for example topical application of irritants or allergens or by injection of such compounds and pathogens, such as bacteria, into the skin (typically to the ear or back of the animal). The symptoms that will develop are inflammation, redness, flaking and thickening of the skin, and skin lesions. Treatments involving novel manipulation of the immune system will then be tested for ability to ameliorate the symptoms of these diseases. The treatments will be delivered either locally by injection/topical administration, or systemically by injection into the blood, or the peritoneal cavity.

Such experiments will typically last one to two weeks and may involve a single injection to induce the disease (or seven daily application of cream to induce the disease), followed by repeated injection (typically two to three times per week) of the treatment. However, a minority of models may extend to 12 weeks to allow for a period of recovery, without

intervention, prior to rechallenge and assessment of treatment efficacy and duration, and assessment of secondary immune responses.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of mice are expected to experience mild to moderate discomfort due to aggravation of the skin, and may experience weight loss during the course of the model. Mice are regularly monitored during studies. The main impact on the animals will be the local symptoms of inflammation. These include redness, flaking and thickening of the skin and possible over grooming. Small areas may also develop lesions of the skin when an **infection model is performed.**

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- 5% subthreshold
- 20% mild
- 75% moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although we undertake many experiments using ex vivo human cells and immortal cell lines, the existing in vitro assays do not accurately mirror the more complex molecular and cellular interactions that regulate immune and haematopoietic cells in vivo, leaving no practical alternatives to studies in whole animals. Despite the initiation of studies (by others) to perform computer modelling of the immune cytokine network these systems do not currently represent a viable alternative to experimental research. In particular, translational aspects of our research, such as the modulation of the immune system, are only possible in an in vivo context.

Using a mouse model of skin inflammation allows analysis of the complex interaction of the skin with different organs in the animal, including migration of subsets of immune cells from the blood stream, lymphatic system and bone marrow. Mimicking the symptoms of

psoriasis and dermatitis involves restructure of the tissue and formation of features such as flaking skin and inflammatory lesions. This process involves numerous cell types, organs and signalling molecules and as such requires the in- vivo tissue architecture in order to develop.

Which non-animal alternatives did you consider for use in this project?

We perform numerous human in vitro assays to both define the function of factors and cells under investigation, and to refine our experimental approach so as to optimise the information gained and predict how the molecule will act in vivo. We have refined an in vivo human allergen challenge model in which skin blisters can be raised at the site of allergen immunisation to give access to fluid and cellular infiltrate for analysis of the response to those allergens.

We are using several in vitro experimental methods to analyse skin tissue and blood from healthy individuals as well as patients with psoriasis, atopic dermatitis and contact dermatitis.

We use imaging and single cell analysis of gene and protein expression of human tissue samples and use this analysis to predict genes and proteins that play a causative role in the development and susceptibility of the skin conditions.

We isolate T cells from peripheral blood in order to identify cells that can interact with skin cells during inflammatory conditions. We have isolated functionally interesting T cell clones and used these for in- vitro analysis of pro-inflammatory, or anti-inflammatory responses.

We utilise human skin obtained from healthy individuals (from discarded tissue from plastic surgery or biopsies) to recreate in vitro tissue that can retain function for a limited period and to isolate cells for functional and investigative analysis. We use skin tissue derived from patients and healthy donors to isolate several types of skin cells that are involved in development of skin inflammation (e.g., Langerhans cells). We can maintain these cells in a functional state for a limited period during which we can coculture them in vitro with T cells to assess the nature of this intracellular interaction and potential contribution to disease. In this way we have tested and refined a number of potential therapeutics prior to in vivo assessment. For example, we isolated and purified over 100 potentially neutralising antibodies and using a human in vitro antigen presentation model we refined this panel to 5 antibodies for in vivo murine investigation.

Why were they not suitable?

The in vitro and ex-vivo techniques we employ are very useful in generating hypotheses regarding the causes of skin disease and form and refine the theoretical basis for developing treatments, but they do not allow for multi-tissue migration and interaction analysis, and development and testing of therapeutic treatments with multi-factorial analysis of the symptoms of disease. Indeed, temporal and spatial factors are missing

from in vitro and ex vivo analyses. For example, immune cells produced in the bone marrow, may require migration to the thymus for maturation, where upon cells migrate to the site of infection, before subsequent migration and development of immune memory to allow rapid recall upon reinfection. This diversity of microenvironments and time-scale cannot be reproduced in a culture dish or isolated organoid. Reductionist reconstructed cellular in-vitro systems also suffer from bias that we introduce by the choice of cell types co-cultured.

Other limitations, with ethical and practical implications, include the frequency and amount of tissue that can be recovered from human volunteers and patients, the limitation of tissue recovery at early stages of the disease- before symptoms develop, and the limitations on the testing of novel experimental treatments. The use of human cell culture and blister analysis offers tremendous insight, but before we attempt to manipulate the human immune system we need significant amounts of preclinical information that help to inform on the likely efficacy and potential side-effects.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals to be used in this project is based on the estimated requirements for experiments in each model as well as the number required to breed and maintain a productive colony.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The group and I have attended courses on experimental design and statistical approaches and will seek further statistical advice from colleagues whenever necessary. We are also aware of online tools such as Experimental Design Assistant (EDA) and G*Power (http://www.gpower.hhu.de/). The web application, Experimental design Assistant (https://eda.nc3rs.org.uk/eda/landing), has been used to rationalise optimal numbers of animals for future experiments. Pilot studies, will be used to test the practicality of experimental design and provide estimates of variability for power analysis to determine future sample sizes.

Shared control groups are used in combined experiments where more than a single question is addressed to reduce the need for repeated control groups. The simplest

example being when using three different blocking antibodies in identical experimental setting, there is only a need for a single isotype control group.

Where appropriate, experiments will exploit a within-sample repeated-measures design to maximise the statistical analysis from fewer animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Best practice is followed for colony management to ensure we minimise breeding of animals that are surplus to our experimental needs. The Establishment has a dedicated colony manager who can advise. There is also a mouse database from which we can source data for our strains that assists in colony management

Pilot studies that determine the parameters for group size calculations will be used to optimise the number of animals used in the experiments.

Collection of multiple tissues at the end of experiments (occasionally storing them for later analysis) will reduce the need to repeat experiments for the purpose of assessing different aspects of the experimental conditions.

Additionally, once characterised, we will make our animals available to the scientific community thereby removing the need to generate multiple animal lines with the same genotype. And we collaborate with labs to provide murine data to their human studies, again reducing the need to regenerate lines.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using several models of skin disease, which differ in the initial stimulus. These include creams containing an inflammatory compound or allergen, or intradermal injection of an allergen or pathogen that causes skin disease. Where possible in the study of skin inflammation, responses to these substances will be primarily localised rather than systemic in order to reduce the overall suffering. Most experiments will be restricted to one to three weeks in order to limit the duration of skin discomfort.

Topical administration is amenable to control through the daily application of cream where the dose can be reduced or stopped to allow skin healing. Where ever possible allergens and irritants will be administered to the ear in preference to the back to reduce the affected area of skin, but for sensitisation and re-challenge experiments the site of skin administration may differ between the first and second administrations (e.g. ear and back). These models facilitate fine control of skin inflammation, in our experience refined application of inflammation inducing agents allows robust detection of immune responses whilst minimising suffering of the mice.

The severity of inflammation can be scored based on the objective clinical Psoriasis Area and Severity Index (PASI). Evaluated parameters include degree of redness, scaling and thickening of the skin. The level of erythema can be evaluated with the help of an optical red scale, and ear thickness is measured without bias using a micrometer. This will allow robust data collection and careful monitoring of the murine experience.

In a few experiments, mice will undergo bone marrow transplant, which involves irradiation that kills the cells of the mouse's bone marrow to be replaced by new bone marrow that is injected shortly after the irradiation. This treatment causes some illness but it is short term (about a week) and the mice recover with no lasting harm. This type of experiment is an important tool for assessing the role of blood cells, and specific proteins expressed in blood cells, in the progression of the disease.

Batches of bacteria or fungi, or irritants/drugs may vary in pathogenicity or toxicity, whilst different strains of mice may vary in their resistance or susceptibility. To reduce the risk of unanticipated suffering we will first employ a low dose group of two mice, a further two mice may be tested at a higher dose and so on until an appropriate dose level is reached. We use a comprehensive monitoring and scoring system to assess the animals throughout experiments. Where possible we will use the shortest models of disease that will yield satisfactory data.

Why can’t you use animals that are less sentient?

Mice are currently the least sentient mammalian species in which these experiments can be performed robustly and reproducibly. Murine immune and haematopoietic systems closely resemble those of humans and many models of human disease have been developed in mice. The mouse also benefits from well-established and robust technologies for transgenic and gene-targeted genetic alteration.

In particular when investigating skin immunology, animals that are less developed evolutionarily e.g. zebra fish and drosophila flies, do not suitably recapitulate human skin biology.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Experimental protocols are formulated to minimise stress or harm to the animals and are based on the observations in pilot experiments, repeated past experiments and published observations.

We continually refine our methods, including changing humane end points, assessing and adding new monitoring techniques (e.g., equipment for measuring ear inflammation), and reducing number of procedures to the minimum necessary for the desired effect (e.g., reducing the number of injections of compound once a sufficient effect has been observed).

We regularly inspect animals to determine their welfare (e.g., take ear thickness and weight measurements and observe changes in their social behaviour and appearance). We use comprehensive monitoring and scoring systems where appropriate to assess the animals throughout experiments.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The design of experimental procedure will follow best practices guidance described by ARRIVE guidelines, local guidelines, and PREPARE guidelines as well published guidelines on skin inflammation models. The NC3Rs and the LASA websites provide invaluable information and our Named Information Officer provides further information regarding refinement with regular emails and can be consulted.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly review the literature relating to animal models and improvement/refinements of models that relate to our work. We will also discuss ways of improving our models with other groups and with the Named Information Officer, Named Veterinary Surgeon and Name Animal Care and Welfare Officers. We also access specific literature on animal welfare through the NC3Rs web site and regular links sent by the 3Rs Information Officer at the University's Home Office Administration Unit. The group and I attend internal 3R’s meetings and talks to keep us informed of advances in 3Rs that can be implemented.

27. Inducing New Neurons for In Vivo Brain Repair

Project duration

5 years 0 months

Project purpose

Basic research

Key words

brain injury and repair, nervous system regeneration, regenerative medicine, cell-based therapy, epilepsy

Animal types	Life stages
Mice	adult, pregnant, juvenile, embryo, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims at developing strategies of generating new neurons in the brain in order to repair or replace damaged, dysfunctional or diseased neurons and brain circuits.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There is a pressing need for effective treatments for a broad spectrum of disease afflicting the brain. This includes neurodegenerative diseases (e.g. Alzheimer's and Parkinson's disease), neurodevelopmental disorders (e.g. autism, schizophrenia, epilepsy) and damage to the brain (e.g. caused by trauma or stroke). In all these diseases neurons have become dysfunctional, damaged or even lost leading to disruption of functional brain

circuits with devastating consequences for affected individuals and society. Our brains have extremely limited ability to generate new neurons and promote self-repair. This project aims to overcome these shortcomings by developing innovative strategies that convert non-neuronal cells in the brain into new neurons that can integrate into brain circuits, with the long-term goal of restoring brain function in human disease.

What outputs do you think you will see at the end of this project?

At the end of this project we will have learnt:

new and more effective ways to convert non-neuronal cells into clinically-relevant neurons; the specific properties of newly converted neurons;

the potential of newly converted neurons to contribute to circuit function and functional restoration.

appreciate differences between non-neuronal human cells and mice cells in their ability to convert into neurons

We will communicate our findings to the scientific community through peer-reviewed publications and presentations at scientific meetings. We will disseminate our research to the general public through non-specialist publications, online material and public outreach events.

Who or what will benefit from these outputs, and how?

In the short term, the main beneficiary will be the scientific community, who will gain valuable knowledge about the mechanisms governing cellular identity, maintenance and plasticity in the brain.

After completion of the project, the translational research community will benefit from protocols and strategies we have developed. These can be used to inform future strategies for cell-base therapies of brain diseases in humans.

In the longer term, we hope that our work will provide a scientific basis for the treatment of currently incurable brain diseases with direct benefits to patients and society.

How will you look to maximise the outputs of this work?

We will maximise our output and impact of our work through:

collaborations with colleagues within our research centre, within the UK and overseas, to complement our own skillset with world-class expertise in the fields of neurodevelopmental sciences and brain disease.

share our expertise and new findings to colleagues to promote complementary research on brain repair.

publish rigorously tested scientific findings that will provide useful information to the scientific community, irrespective of whether they support or falsify our original hypotheses make raw data and protocols widely available to ensure our work can be validated by other research groups.

Species and numbers of animals expected to be used

- Mice: 8500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will use mice to study the possibility of generating new neurons in a brain where this does not normally happen spontaneously. Unlike mammals (such as mice and humans), lower vertebrate species, such as fish or amphibians, have a natural ability to regenerate neurons throughout life.

Therefore, to model the challenges of generating new neurons in a brain akin to humans, we need to use animals that lack the ability for natural neuron regeneration, such as mice.

Importantly, mice have similar brains as humans, and like us humans possess a cerebral cortex, which can be afflicted by many devastating human diseases and, for this reason, is a main focus of our project. Furthermore, mice are genetically very similar to humans. This means that the mechanisms of action for the strategies we develop to convert non-neuronal cells into neurons in the brain, are likely to be directly relevant to humans.

Our work needs to be conducted in animals at a time when developmental birth of neurons has come to an end. In mice, this process is complete at the time of birth, therefore it is necessary to conduct our work after birth. Human brain disease occur throughout life. Some disorders are typically diagnosed in early life, including many neurodevelopmental disorders (e.g. autism, childhood epilepsy). Others may manifest in adolescence or early adult life, such as schizophrenia and other mental health disorders. Still others may typically affect us in later life - for example, Alzheimer's disease and stroke. Therefore, it is important that we consider a range of life stages when investigating the potential of our strategies to convert non-neuronal brain cells into new neurons. Additionally, it is important to allow time for our newly induced neurons to mature and integrate into brain circuits. We also need to assess the long- term survival of these new neurons and their therapeutic effects on the organism.

For these reasons, we need to study mice over a range of life stages from birth through to adulthood.

Typically, what will be done to an animal used in your project?

In order to generate new neurons in the brain from non-neuronal cells, we need to deliver certain genes into the non-neuronal cells (e.g. astrocytes). We do this as part of a surgical procedure, where the mice are anaesthetised and we inject vehicles carrying genes into the specific part of the brain we are studying. Almost all mice in this project will have a surgical procedure such as this.

A large minority of mice will also have a second procedure. This can be for one of several reasons. For example, in order to study how the new neurons integrate into the brain, we can place a small glass window in the skull, which allows us to directly observe the new-born neurons as they grow, change shape and become active under a microscope. Alternatively, a second surgical procedure may be required to inject human astrocytes. This allows us to make sure that our research is as applicable to humans as possible. Less commonly, we may perform a surgical procedure to cause a localised and controlled lesion that in some specific way emulates human brain injury or disease (e.g. epilepsy).

Doing this is important to test if generating new neurons might be a useful treatment in brain disease or injury. However, these models of brain disease are typically mild and only account for a small proportion of mice in our project.

Therefore, a typical mouse would undergo either one or two surgical procedures where they are fully anaesthetised. In some cases (i.e. where we fit a glass window to observe the brain) we would place these mice under the microscope several times over a period of a couple of weeks. Sometimes, mice may need a few injections under their skin or in their abdomen to activate genes.

What are the expected impacts and/or adverse effects for the animals during your project?

We have taken care to design our experiments to have the minimal impact/adverse effects on the animals whilst still being able to answer our scientific questions.

Most experiments in this project require mice to have at least one surgery, however, and these do carry risks. Pain, anaesthetic overdose bleeding and infection are the most common side-effects. We work with the veterinary surgeon to make sure we give the correct dose of painkillers and anaesthesia meaning that with good surgical practices these happen only rarely. Mice are reviewed daily, so side- effects are picked up quickly and most can be easily treated with simple interventions (e.g. giving extra painkiller medication) within 24 hours.

A very small number of mice in this project will be used to answer questions about whether creating new neurons can be a treatment for epilepsy. These mice have a specific injury that causes electrical discharges in a part of their brain. They do not have convulsive seizures but sometimes they might show some abnormal behaviours (such as stopping

mid-activity). These aren't harmful or painful for the mice, but they can occur throughout life.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 50% of animals in our project will experience mild severity (mostly related to general husbandry and breeding of the animals, some of which have an injection(s)).

The other half of animals will be those who have at least on surgical procedure, and these would be classed as experiencing moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The brain is the most complex organ in the body, a fact that can be attributed to the plethora of different types of cells and their multiple, highly specific interactions with one another. These include exquisitely specific communications between neurons within a given brain area, as well as those across brain areas. Moreover, these are complemented by interactions with various kinds of non- neuronal support cells, such as astrocytes, oligodendrocytes and their progenitors, microglia and other immune cells, and cells of the vascular system (e.g. pericytes and endothelial cells). To make matters even more complex, these interactions undergo change throughout development of the organism and depend on the health and life experience of the organism. In previous and ongoing work in our laboratory, we have successfully developed strategies to reprogramme non-neuronal brain cells into neurons using cells in a culture dish, revealing many important aspects of the underlying biology.

However, we have also learnt that the complex environment of the brain poses additional barriers to the conversion that can not be recreated in a dish. Furthermore, we are now trying to understand whether new neurons that we generate are capable of integrating with other neurons in the brain and become recruited into functional brain circuits. Finally, we want to determine if these new neurons can provide beneficial effects to malfunctioning brain circuits, such as those we see in human brain disorders. This requires us to conduct

experiments in suitably complex animal models of healthy and disordered brain function and measure the functional consequences of induced neurons on neural circuit activity.

Which non-animal alternatives did you consider for use in this project?

1. Cell Culture - growing human or non-human cells in a laboratory dish

2. Brain organoids - 3-dimensional cell aggregates derived from pluripotent stem cells

3. Computational models

4. Human volunteers/patients

Why were they not suitable?

1. Cells in culture have differences in gene expression and states of maturity and, therefore, do not present the same barriers to cell-fate conversion strategies encountered in living mammals. They also do not mimic the degree of complexity needed to study the importance of the local cellular environment or integration of new neurons into brain circuits.

2. Brain organoids are a new technology, which we work on in the lab as well as using animal models. They succeed to mimic a bit more of the cellular complexity that exists in the brain. However, many cell types and structures are missing and the overall architecture remains immature. They are also very restricted with respect of modelling complex human diseases or behaviours. We closely follow this advancing field and wherever possible would prefer to work this system over animals. Currently, this system is not suitably advanced to meet the aims of the project proposed here.

3. There are no suitable computational models that capture the dynamics of gene regulatory networks and their perturbation during cell conversion and across several cell types within the complex context of functioning brain tissue.

4. At this stage, glia (non-neuronal cells in the brain) to neuron conversion is at a very early stage of exploring its feasibility in the live brain. Our studies are an absolute prerequisite for any potential clinical translation in the future.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have experience working with mice for research and can look at the exact number of animals that we have bred during a year. If we breed similar numbers per year over the next 5 years of this project, we estimate we will breed between 5500 and 7500 mice. However, since our research team is likely to

expand during the next 5 years, it may be the case that there may be more mice bred in the next years than years that have passed. Therefore, we estimate that this project will require around 8,500 mice to be bred during next 5 years, however, not all of these will be subjected to scientific experiments, many are used in breeding and colony maintenance.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We design our experiments in such a way to keep animal numbers used to a minimum that still provide robust statistically valid answers to our scientific questions. We request that all members of the lab visit the NC3R's website (https://www.nc3rs.org.uk/the-3rs) and use this and other appropriate tools to calculate appropriate sample sizes when designing their experiments.

We also take steps to maximise the amount of information gained by each animal. Examples of this include the use of some imaging modalities which allow data to be collected over time in the same animal to be taken (rather than, for example, culling cohorts of animals at specific time points). Our work is increasingly trying to invest in and develop tools and training that will help us to collect this sort of data so we can reduce overall animal numbers. We have built this into our experimental designs. In these scenarios, however, it is important to ensure that reducing the number of animals used is balanced against any additional suffering that might be caused by repeated data collection on a single animal. Once animals are humanely killed and tissues collected, we can also perform multiple different types of analysis on a single mouse brain. This allows us to collect lots of information from one mouse, rather than needing to use a separate mouse for each type of analysis.

Sharing data and resources (e.g. animals, tissues and data) between research groups and organisations can also contribute to reduction. We use genetically altered mice lines that have established protocols (e.g. drug dosing protocols) to avoid needing to use animals to create our protocols from scratch.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Whenever we are trying a new viral tool in the lab or a new technique, we conduct pilot studies on a very small number of mice to ensure protocols are working well. This allows us to avoid subjecting larger numbers of animals to a treatment before knowing if the tools and techniques are working.

Very often, we use genetically modified mice to study specific cell types in the brain. If an animal has only one copy of a gene, then there is only a 50% chance of their offspring having the gene we need for conducting our scientific experiments. This means that around 50% born may not be suitable for particular experiments. It is usually more efficient to have mice that have two copies of the gene (homozygous). That way, 100% of the litter have the gene of interest and avoids breeding additional mice. We use this wherever possible, however, there are many situations where it is still necessary to maintain mice that have only a single copy of the gene. Although we often need genetically altered animals to perform experiments, some of our work can be done in 'wild type' (non-genetically altered) mice. We can often, therefore, use the siblings of the genetically altered mice to conduct experiments - saving on the unnecessary breeding of additional mice.

We encourage researchers to share brain tissue or other samples that they have in surplus so that information gathered from a single mouse can be maximised.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Work on this project is exploring strategies to generate new neurons in the brain, which may one day help develop treatment for human brain diseases. To do this we deliver genes to specific cells in specific parts of the brain. We mostly do this by using viruses that have been genetically modified to remove harmful viral genes and prevent their replication but can still express the genes we want to study in the cells that they infect. Delivering these genes involves an injection into the brain as part of a surgical procedure. To be able to directly visualise new-born neurons in the brain and explore how they integrate into local brain circuits, we make use of techniques to implant small transparent windows and other devices that can help to measure neural activity. These procedures are well-established and are performed under anaesthesia and with attention to making sure mice receive appropriate doses of painkillers and general post-operative care when the wake from any operation. Wherever possible, we try to only perform one operation on any one mouse. Although there are several situations where two, or at most three, operations are required for scientific reasons. We make sure mice are allowed to fully recover between operations.

Since we are trying to develop strategies that may one day be used to treat human disease, it is important to study the role of injury and disease on our reprogramming and

vice versa. To do this, we use two approaches. One is to make a small cut in the cortex of the mice during a surgical operation. Because this cut is very small, localised and controlled the mice do not show any abnormal behaviour or signs after this injury but still allows us to get crucial scientific information. Our other approach is to study whether newly reprogrammed neurons could be beneficial in treatment resistant epilepsy. We use a well-established model where a chemical is injected into the hippocampus of mice, which causes some neurons to degenerate, causing a form of epilepsy called mesial temporal lobe epilepsy (MTLE). Because the damage in MTLE is restricted to the hippocampus, it is called a focal epilepsy. The advantage of using this model is that the mice do not have convulsive seizures as the seizure activity is 'focal' at the hippocampus and surrounding tissue and does not spread throughout the brain. This means that the mice can live almost behaviourally normal lives (although they can have some behavioural pauses and repetitive movements) while still allowing us to study the benefit of reprogrammed neurons in the context of epilepsy. The MTLE and brain injury mouse models account for only a small part of our work.

Why can’t you use animals that are less sentient?

Many non-mammalian animals such fish and amphibians show a much greater ability to regenerate their nervous system than mammals. Since we are ultimately interested in developing treatments to repair damaged or dysfunctional human brains, to have any relevance, we need to study this in a scientifically appropriate (mammalian) species. Although all our surgical procedures are performed under anaesthesia, regenerating neurons from glia in the brain takes time (weeks to months).

Therefore, we can't study conversion of glia to neurons in terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We regularly review and update our protocols for anaesthesia, analgesia and any drugs that we may administer to animals with the veterinary surgeon to ensure that the route and dose of any treatments are optimised to keep any pain or suffering to a minimum during the experiments that we do. We ensure that mice are kept warm, observed and generally cared for after any surgical procedure and that they make a full recovery before being returned to their home cage. During operations mice have their temperature maintained, breathing monitored and pain reflexes checked regularly to make sure that they are well and pain free during all procedures. New researchers are trained under supervision by experienced researchers to make sure they are competent with any new procedure before being allowed to work independently. Mice are visually checked by animal staff daily in our animal unit to make sure they are well. After a procedure, researchers perform additional checks to ensure recovery is complete, administer any necessary post-operative pain relief and that any implanted surgical devices are not causing discomfort to the animal.

When awake animals are used during imaging or data collection, we ensure that the mice are gradually habituated to the experimental/microscopy environment (usually in brief sessions over several days) before any real data is collected. This reduces any stress the mice may experience by the act of collecting data and they are usually very quick to relax when well habituated.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Researchers will be familiar with and expected to meet the criteria laid out in the latest published ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

We follow best practice advice laid out by NC3Rs at: https://www.nc3rs.org.uk/3rs-resources/anaesthesia and expect new and experienced researchers to complete the e-learning resources and implement these practices.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will work with our Named Training and Competency Officer on our Establishment licence to ensure that information about advances in 3Rs (and training opportunities) are being communicated and disseminated to researchers working on animals. We are setting up regular scheduled meetings with researchers performing animal work to come together to discuss local issues with animal work and foster a culture best practice in refinement techniques. These will be a forum for discussion on the practical implementation of any advances in 3Rs in our project. As well as completing all mandatory training, any new researchers in the group are expected to set aside time to visit the NC3Rs website to read/watch all aspects relevant to our protocols, as well as general best practice in husbandry, experimental design, euthanasia and surgical procedures.

28. The Developmental and Heritable Basis of Adaptive Variation in Fishes

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

adaptive divergence, climate change, phenotypic plasticity, genetic mutation, development

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, adult, juvenile, aged, pregnant
Gasterosteus aculeatus	embryo, juvenile, neonate, adult, aged, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project will use fish to understand the genetic basis and developmental factors responsible for adaptations. A range of species will be used to address different aspects of adaptation including changes in metabolism and bone development.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Natural selection can't work without variation in traits (morphology/anatomy, behaviour, physiology) to sort. Therefore, we address a number of important questions including how the environment contributes to trait variation, and how development and genetics contribute to trait variation. This has broad importance for research on the effects of climate change on natural populations, and the use of fish as model systems for m medical topics such as metabolic research (including diabetes and obesity), as well as common birth defects such as cleft palate (i.e. craniofacial research). The science involved will bring forward an understanding of how variation arises before selection in a way that is unique in the field.

What outputs do you think you will see at the end of this project?

The projects will result mainly in a number of publications focused on the genetic and developmental basis of trait variation in fish. We expect no commericial products to arise. We hope that we will open new doors to the study of human diseases and the effects of climate change on natural populations and start to get a handle on the genetics and environmental factors involved.

Who or what will benefit from these outputs, and how?

This will benefit fish biologists and evolutionary biologists. In the short-term the research will be of interest to other academic scientists but over the longer term we think the findings will be relevant to medicine as well to biologists interested in how populations of cold-blooded ectotherms will respond to warmer habitats as a result of climate change. This will include research on facial variation and bone development, as well as metabolism. The work will develop fish as a model system for these human syndromes and potentially shift research towards them over time.

How will you look to maximise the outputs of this work?

The work will be published in journals, and presented at national and international conferences. Press releases will be provided for some outputs to enable news organizations to pick up on our findings.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 2500 Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Fish are an extremely variable group, both within and between species which makes them applicable to a wide range of questions. Natural populations of fish are well known to exhibit adaptive variation in head and body shape, behaviour, and physiology. This occurs naturally as adaptations, but it is also the case that populations are facing new challenges in a changing world and will likely need to change. Fish also share a surprisingly strong genetic identity with humans (many traits have the same genetic basis in both humans and fish) making them relevant to human health conditions and are highly suitable to experiments. Many species are also well studied in terms of developmental stages and the events that occur as they grow (i.e. they possess a published ‘developmental series’) making assessments of change straightforward. This lets us use fish to target many key stages such as when the jaw first forms, or metabolism and growth slows.

Typically, what will be done to an animal used in your project?

Most fish would be reared under stable conditions with mild experimental procedures being applied such as changes in diet, feeding, flow, or temperature. However, some focal individuals would undergo injections of compounds to aid in individual identification, or for experiments involving assessments of physiology. Some fish would also be provided with exposure to pharmacological compounds (at larval stages) in the water, targeted at testing the effects of molecular pathways. Finally, we would occasionally perform genetic editing techniques and rear fish to examine the phenotypic effects. These genetic edits would be targeted at genes involved with adaptation and thus are unlikely to have negative effects on fish.

What are the expected impacts and/or adverse effects for the animals during your project?

Our experimental procedures involving injections would involve momentary pain under anaesthesia lasting seconds to minutes.

In some cases we would also need to reduce feeding levels for periods of 6-8 weeks to induce metabolic effects.

Blood and fluid extractions would also be a potential source of temporary stress lasting on the order of minutes to hours.

The use of anaesthesia for some procedures, while reducing stress in many cases, is likely to have effects on behaviour (unbalanced swimming, laboured breathing) lasting a few hours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mild (~80% of subjects) - Moderate (~ 20% of subjects)-

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of live fish is the only way to experimentally test and understand the basis of adaptive trait variation. Many fish species are new study systems for experimental approaches and therefore important insights about their specific adaptations have yet to examined. A non-animal alternative would not provide a means to make discoveries about the developmental and genetic basis of adaptation.

Which non-animal alternatives did you consider for use in this project?

We could not consider non-animal alternatives for this project. This is because we are interested in adaptive traits are within fish and need to be assessed them directly. These are not well understood in general, especially for fish, and thus in silico approaches would not be feasible.

Why were they not suitable?

Non-animal alternatives could not provide information about how specific populations respond to environmental change. As we are interested in whole animal responses and the genetic factors involved, our project requires animal subjects.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated our numbers on the basis of current practises in the field. For example, for G. aculeatus a recent study (https://onlinelibrary.wiley.com/doi/pdf/10.1111/mec.13965) shows that for fish an experiment to relate genetic variation to trait variation the typical experiment uses 300 individuals.

However, in our case we will use two independent populations and ages which each double our needs to 1200 individuals for this part of the project. Similarly, we have investigated traits previously and found that a sample size of 10-40 individuals is required (10 for molecular measures, 40 for outward morphological traits). This approach will make the work acceptable to the field, making the use of these animals worthwhile.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have planned a set of novel experiments on understudied traits. For statistical analysis this precludes knowing the levels of variation for each trait we plan to examine making it difficult to provide precise numbers. However, the power of our experiments to detect statistical differences due to temperature, population, dietary, and age effects will be enhanced through the implementation of a randomized block design including factors such as population, rearing temperature, and species/ecotype as fixed blocked effects. We will also regularly employ the use of generalized linear mixed-models to limit the potential effects of pseudoreplication using individuals as a random effect (some fish will need to be raised within the same experimental unit). Overall, this will enhance statistical power, as observations will be made at the individual rather than group level. This means that individuals could be measured more than once without any statistical compromise being made.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Reduction will also occur through the measurement of multiple traits on the same individual when sampled (e.g. fat level, body composition, appetite level, morphological shape). Also, some of the methods we use to assess body composition (micro-CT scanning) will enable morphological variation to be assessed in 3D for future projects. The scanning will take place locally with a collaborator who is in possession of a micro-CT scanner.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The fish species we intend to use are ideal models for a range of questions. This includes questions surrounding evolution and development, but extends to clinically relevant variation, and the impact of climate change on natural populations. What is occurring in fish is that some adaptations from nature can actually mimic traits found in human disease whereby ‘screening’ has been done by natural selection instead of lab researchers. So, while clinicians usually focus on mutant lines screened for traits of interest, and which may suffer from inbreeding, or generally poor health our fish are robust. This is because the traits of interest are part of an adaptive process that limit the negative effects associated with a 'disease' phenotype. This is a special aspect of adaptations that standard lab models may never offer. Ultimately, this should lead to a more powerful way of studying disease and require fewer animals overall, and the study of traits that do not compromise the health of the animals. This will provide a better approach relative to the use of highly inbred lines with large, often lethal, mutations.

We will use standard lab assays for the measurement of metabolic traits (Western blots, qPCR) and standard techniques for the measurement of morphology. The fact that fish of a single species can also live and reproduce in a range of temperatures is making them increasingly relevant for understanding the impacts that climate change could cause. The populations of sticklebacks we have identified living in warmed waters fish this very well and can help us look into a potential future situation of how adaptations occur in a warmed world.

Why can’t you use animals that are less sentient?

We will use adult fish mostly, but also their larvae prior to independent feeding to understand development. Thus, we will use these species at less sentient stages. However, when studying the effects of advanced aging, or traits that emerge at later life stages it is simply impractical to consider a less sentient set of species within the vertebrates. The fish that we rear will largely be kept for morphological/anatomical analysis and will allow them to serve as study subjects in the current and future projects.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have existing bespoke protocols for anaesthesia for each of the species that we will continue to monitor and adjust. The aquaria we use will also be equipped with enrichment materials such as rocks, structures, plants as appropriate to the species. We have found that this leads to seemingly calmer fish. Given the variety of cichlid species we rely on, a

set of references regarding their preferred diet, with the ability to provide herbivorous or carnivorous foods. We will even provide these foods at different depths in accordance with the ecology of a given species. Temperatures and water parameters are also to be kept in line with those found in nature. Together these aspects should reduce stress and recovery times from any potential impacts of procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Westerfield, M. (2000). The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio). 4th ed., Univ. of Oregon Press, Eugene.

Ribbink, and B. J. Sharp. (1983). A preliminary survey of the cichlid fishes of rocky habitats in Lake Malawi. South African Journal of Zoology.

Schultz E. (2014) Juvenile Threespine Stickleback Husbandry: Standard Operating Procedures of the Schultz Lab

https://production.wordpress.uconn.edu/fishlab/wp-content/uploads/sites/116/2014/07/CompleteSTBKhandbook_31jul.pdf

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will be in regular contact with our local NACWO as the fish are kept under experiment. I will also be in regular contact with our local vet and belong to a mailing list of project licence holders that will continually inform us of changes to legislation. Regular checks of NC3Rs and Norecopa websites will be made for confirmation and updates to possible refinements.

29. Pathological Calcium Leak and Abnormal Heart Rhythm

Project duration

5 years 0 months

Project purpose

Basic research

Translational or applied research with one of the following aims:

- Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

cardiac, arrhythmias, sudden death, catecholaminergic polymorphic ventricular tachycardia, heart disease

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Patients with heart disease are at greater risk of dying suddenly due to abnormal heart rhythm. This project seeks to understand the mechanisms linking "leaky" calcium channels in the heart and arrhythmias and work towards developing new treatments to prevent sudden death.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Sudden cardiac death is a major global healthcare problem, estimated to account for up to 20% of deaths in Western societies, or more than ~7 million deaths globally per year. It is not fully understood how arrhythmias start in the heart, however loss of control over cellular calcium levels is thought to be an important contributor. This project will firstly generate scientific knowledge of the mechanisms linking calcium and arrhythmias in heart disease. By better understanding the mechanisms of disease, this could influence the current clinical management of patients by opening up new opportunities using already available treatments. This improved understanding will guide the development of new therapies for heart disease. In the longer term this research could improve the quality of life and reduce premature mortality in a large and growing section of the global population.

What outputs do you think you will see at the end of this project?

This project will generate new scientific insights into the cellular origins of arrhythmias in the heart. This will improve our understanding of how arrhythmias begin and reveal new opportunities for intervening. We will develop new experimental treatments and validate their efficacy at preventing arrhythmias in a small animal model of heart disease. The findings will be published in peer-reviewed journals and presented at conferences. Eventually this work could lead to new drugs and treatments for patients with heart disease.

Who or what will benefit from these outputs, and how?

In the short term, clinicians and basic scientists will benefit from the improved understanding of the cellular events leading to arrhythmias in the heart (1-2 years). This could encourage a re-thinking and re-evaluation of the treatments currently offered to patients with heart disease, and prompt trials of existing drugs that are not currently used for this purpose but which may have a positive effect preventing arrhythmic events. Therefore, in the medium term (3-5 years) this could benefit patients that currently have heart disease by improving the way their condition is managed. In the long term (5-10 years), the anti-arrhythmic treatments developed here could lead to clinical trials and new treatments for a broad range of patients with heart disease.

How will you look to maximise the outputs of this work?

We will work closely with national and international collaborators with expertise in different areas of the project. These collaborators will provide technical facilities, materials and knowhow to ensure the project success and the ability to complete all objectives during the project timeframe. In addition, we will establish connections with clinical cardiologists to hone the clinical aspects and maximise the potential for this research to translate into patient benefit. Our results will be submitted for publication in esteemed peer-reviewed journals with high readership to maximise its visibility.

Species and numbers of animals expected to be used

- Mice: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mouse is the species of choice when genetic manipulation is required to mimic human disease and is the only mammalian model used so far to replicate the inherited human hearts disease catecholaminergic polymorphic ventricular tachycardia (CPVT). No larger animal models of CPVT such as rat, rabbit or pig are available. This project requires regular access to fresh isolated heart muscle cells (myocytes). Mouse cardiac myocytes share the excitation-contraction coupling processes of human myocytes that make them a suitable model species for this investigation. There are no non- animal sources of adult myocytes. Non-animal alternatives such as stem cell derived cardiac myocytes and zebrafish cardiac myocytes do not have the same structure and function as adult mammalian cells and so cannot be used for this purpose. The mutation being studied results in a "leaky" calcium channel which is characteristic of CPVT caused by other mutations and common pathologies, such as heart failure. A main advantage of using this model is that it does not require surgical intervention and mice do not experience a detrimental phenotype. Therefore these results should be applicable across a wide range of human disease, without inflicting unnecessary suffering. CPVT is typically diagnosed in childhood and with the best currently available treatment patients can live into adulthood. Therefore mice will be used at approximately 4 months old, representing the equivalent of early adulthood in mice.

Typically, what will be done to an animal used in your project?

Procedure 1: Genetically altered mice with a mutation in a cardiac calcium channel will be bred and maintained for experiments. The majority of mice will undergo no further procedures and will be killed at approximately 4 months old for collection of heart tissue to generate single heart muscle cells.

Procedure 2: A small number of mice will have radio transmitters surgically implanted under the skin to record and transmit electrical heart signals (ECGs). The ECG will be recorded wirelessly during anobservation period of up to 4 weeks in conscious, freely moving mice, to detect the incidence of irregular heart rhythm. Arrhythmias in CPVT patients typically occur during periods of increased emotional or physical stress when levels of adrenaline increase. To simulate emotional stress in mice, we will briefly and intermittently blow warm air on the mice using a hairdryer under close supervision. Once the observation period is complete, some mice will further undergo a cardiac stress test

(injection of adrenaline and caffeine) under terminal general anaesthesia to establish their susceptibility to arrhythmia.

Procedure 3: We will investigate whether modified proteins can prevent arrhythmias in CPVT mice and provide therapeutic value. To do this, some mice will be injected via the tail vein with a non-replicating and non-pathogenic genetically engineered virus that will deliver the genetic material required to make therapeutic protein the heart. We will investigate whether regulatory proteins are protective against arrhythmias by recording the ECG during normal living and during the cardiac stress tests described in Procedure 2, and by isolating cells from the heart to measure their function (as above).

What are the expected impacts and/or adverse effects for the animals during your project?

Mice with the mutation under investigation do not experience a harmful phenotype in normal housing conditions therefore genetic manipulation alone (as is the case for the majority of mice used) is not expected to produce adverse effects. Surgical implantation of miniature ECG transmitters may cause localised trauma and discomfort that will be mitigated by post-operative analgesics. Surgery carries a risk of wound infection that will be mitigated by surgeries being performed by a skilled surgeon, maintaining sterile conditions during surgery and administering post-operative antibiotics. There is a risk of disrupted wound healing due to rodents chewing their sutures; if this occurs new sutures will be placed. Mice are expected to recover completely within 7-10 days post surgery as shown by a recovery of post-operative weight loss and full recovery of mobility. The virus used to express therapeutic proteins in the heart evokes minimal immune response and should not cause adverse effects. At the end of experiment mice will be killed humanely using an appropriate approved method by qualified and skilled practitioners.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sub-threshold - breeding of genetically altered animals - 50%

Moderate - ECG and protein expression studies - 50%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mouse is the only mammalian species available as a model of the human disease CPVT. Mouse cardiac muscle cells are structurally and functionally similar to human cells which is necessary for this research. These similarities make mouse a suitable model species for this investigation. Non-animal alternatives such as stem cell derived cardiac myocytes do not have the same cellular structures and lack important ion channels compared to mammalian cells and therefore cannot be used for this work.

Which non-animal alternatives did you consider for use in this project?

Induced pluripotent stem cell derived cardiac myocytes (iPSC-CM), human endothelial kidney (HEK) cells

Why were they not suitable?

Although CPVT research has been conducted in iPSC-CM and transgenic zebrafish models these cells have major dissimilarities compared to human heart muscle cells that are incompatible with achieving the aims of this project. Mammalian myocytes possess membrane structures, called t-tubules, that create a localised environment near the calcium channel that strongly influences its behaviour. iPSC- CM and zebrafish myocytes are completely absent of t-tubules and therefore lack this important aspect of calcium regulation. The amplitude and timecourse of calcium released during each contraction is very different in iPSC-CM and zebrafish to human and mouse myocytes, due to different expression levels of calcium regulating proteins and ion channels, and therefore the relationship between calcium and membrane voltage and the relative importance of different arrhythmia mechanisms will be different in these species. Another main difference is that human or mouse ventricular myocytes do not contract unless electrically stimulated or are exhibiting spontaneous activity (the cellular equivalent of arrhythmias in the heart), whereas iPSC-CM do spontaneously contract.

Where possible, e.g. for biochemistry studies, non-animal HEK cells expressing CPVT mutant protein will be used as a suitable alternative to animals because cell structure is not a concern for these experiments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal usage numbers are based on extensive prior experience in this type of research. This includes estimates of the volume of data that can be collected from each animal, the variability in data, and the number of replicates needed from each animal to detect statistical differences between groups. The numbers account for the fact that a single animal can generate data for multiple experiments (because the heart is dissociated into millions of individual cells).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

There is no difference in the prevalence of CPVT between men and women, therefore both male and female mice will be used for this study reducing the number of mice needing to be bred. A principal technique this project will employ involves separating the heart into millions of isolated muscle cells (myocytes) that can be experimented upon separately. This greatly reduces the number of animals used because a single heart can generate data for multiple experiments. Myocytes are normally viable for up to 9h after cell isolation however, for some experiments myocytes will be maintained in special cell culture conditions for up to 48h allowing data collection over multiple days. Where surgery is involved, the experiments are designed to extract the maximum data from each animal e.g. in addition to recording ECG during normal activity in conscious freely moving mice, the arrhythmia susceptibility will be measured in the same mice under terminal anaesthesia. The hearts from these mice will then be used for cell isolation, histological analysis, or protein analysis. For protein biochemistry experiments, human endothelial kidney (HEK) cells will be used as a non-animal source of RYR2 protein. Frozen tissue and cells will be stored for use at a later date. The experimental data obtained from these distinct lines of inquiry will be incorporated into computer simulations of cardiac myocytes to improve their accuracy and predictive power, which will in turn reduce the need to use animals in future.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

I will use the expertise of the Biological Services team to ensure an efficient breeding strategy is used, due to their experiences with breeding transgenic animals. Data gathered during pilot studies were used to estimate the number of animals needed to detect statistically significant differences between experimental conditions and these estimates will be continuously monitored and updated if needed.

We have tissue sharing agreements in place that mean tissue and organs that would otherwise be disposed of can be used by other research groups wishing to use them.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The primary aim of this project is to identify new treatment approaches to reduce arrhythmias and sudden cardiac death triggered by abnormal intracellular calcium. We will use a transgenic mouse model of the human arrhythmia syndrome CPVT to investigate the efficacy of new possible therapies. The most conspicuous difference in CPVT cells is an increase in spontaneous calcium release, which also occurs in highly prevalent heart failure and other cardiomyopathies. A major advantage of using this model compared to other models of heart disease/failure is that no surgical intervention is required to produce a pathological calcium phenotype because the causative mutation is present from birth. The mice do not experience a harmful phenotype during normal living therefore this reduces the pain, suffering and distress experienced by animals. It also reduces the variability between animals thus reducing the number of independent observations needed to detect differences in experiments. We will use wireless ECG transmitter implants allowing recordings to be made in conscious freely moving mice, without the need for restraint. We will use a modified adenoassociated virus (AAV) to express regulatory proteins in the heart of living mice. This causes less immune response in animals and preferentially expresses protein in the heart. The use of AAV is therefore a refinement compared to older virus technologies. Using intermittent warm air from a hairdryer as an unfamiliar stimulus is a painless way to elicit mild emotional stress and increased heart rate in mice.

Why can’t you use animals that are less sentient?

This project requires a source of mature cardiac myocytes that possess similar calcium handling and electrical properties to human. Mice at a more immature stage and less sentient species such as zebrafish have less developed cellular structures and different calcium and electrical properties making them unsuitable. ECG telemetry implants, although small, do require a minimum size of mouse be used therefore younger animals are not suitable. Anaesthesia affects cardiac parameters, such as heart rate, which will affect arrhythmia susceptibility, therefore conscious recordings are the preferred method.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice with ECG transmitters will be housed with non-instrumented companions to minimise stress due to loneliness. Mice will be closely monitored during recovery from surgery to ensure they are eating and their wounds are healing properly. Welfare assessment score sheets will be completed for each operated animal and record any signs of adverse effects

or suffering allowing action to be taken accordingly, such as adjusting pain medication or humane termination. Cardiac-specific expression of proteins can be achieved by intrathoracic injection of adenoassociated virus carrying a transgene, but this is traumatic and carries the risk of lung puncture or cardiac rupture. Instead, we will used a refined approach by using a virus that specifically targets the heart and that is injected via the tail vein using single-use needles. This will improve the efficiency of infection of the heart (because the virus will circulate around the entire heart, not just the peripheral surface) and reduce trauma, pain and risk of transmitting disease.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the NC3Rs Refinement resource hub for updated procedures we could implement

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep up to date with news posted by the NC3Rs on their website, subscribe to the newsletter and regularly check the resource hub for advances in relevant techniques. We will stay in regular communication with Named Persons and animal technicians to stay up to date with developments and improvements we could make to improve animal welfare. We will receive emails from the NC3Rs regional programme manager and participate in seminars and symposia.

30. Enhancing the Effectiveness of Oncolytic Virus-Induced Cancer Immunotherapy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, cancer immunotherapy, oncolytic viruses, metastatic disease, relapsed disease

Animal types	Life stages
Mice	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to test the efficacy of oncolytic virus based anti-cancer therapies and gain a better understanding of the regulatory systems that exist to prevent cancer therapies from working. An improved understanding of the cancer environment will allow us to design novel treatments to eradicate cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We have developed novel oncolytic virus-based therapies for the treatment of cancer using human in vitro systems. However, before clinical translation can be considered it is important to validate the efficacy of these approaches using animal models that recapitulate the complexity of the tumour microenvironment and provide host cellular interactions that could influence anti-cancer responses.

Different types of cancer, or indeed cancers of the same tissue origin, have developed a range of strategies to prevent chemotherapy and/or immunotherapies from working. Moreover, the challenges that exist for the successful elimination of cancer at different stages of disease (e.g. primary cancers, metastatic cancer or relapsed cancers that have re-occurred after initially successful therapy) may also be different. Gaining a better understanding of the strategies used by cancer cells to survive will help us develop combination treatments that are effective against disease. This is important as many forms of cancer remain incurable and novel and effective therapies, that are safe and well-tolerated, are urgently required.

What outputs do you think you will see at the end of this project?

We expect multiple outputs to be obtained by the end of this project. For example, we will identify mechanisms that are used by different types of cancers to prevent therapies from working and develop novel treatments that can be translated into clinical trials to improve patient outcomes. Throughout this project, we will disseminate our findings by publishing data in peer-reviewed scientific journals and by presenting our work at scientific meetings, including local seminars and national and international conferences.

Who or what will benefit from these outputs, and how?

In the short term (within 3 years), researchers working in the field of cancer research or cancer immunology will benefit from these findings through knowledge gain. In the longer term (5-10years), we hope that the results obtained from animal models will be translated to cancer patients and that patients will benefit from the novel therapies identified (e.g., through increased patient survival and/or the development of safer and better tolerated treatments). The clinical application of safe and well- tolerated treatments will ultimately increase the quality of life for patients undergoing treatment.

How will you look to maximise the outputs of this work?

We will publish our research in high-quality peer-reviewed journals and work collaboratively with academic and clinical colleagues at a local, national and international level, to share our experience and expertise. We will also maximise outputs through the dissemination of results at relevant scientific meetings and by the delivery of seminars locally or at other universities.

Species and numbers of animals expected to be used

- Mice: 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use established mouse models of a range of cancers (including ovarian cancer, colorectal cancer and multiple myeloma, amongst others) to characterise the cancer microenvironment and test the efficacy of novel treatments. Mouse models will be used because laboratory studies using cancer cell lines or patient samples do not (i) recapitulate the full repertoire of complex interactions that occur within the cancer environment or (ii) provide information about optimal drug scheduling and delivery routes.

We will use both juvenile (5-10 weeks) or adult mice (>12 weeks) to test anti-cancer agents. This will allow us to determine whether there are differential responses to treatment depending on the age of the animals used. The age of mice at the start of each study will be dependent on the specific cancer model and aim of the experiment.

Typically, what will be done to an animal used in your project?

Animals will be injected with tumour cells at the start of each study and then treated with therapeutic agents. Therapeutic agents could be delivered by injection into the bloodstream, the tumour or the peritoneum. Additionally, therapeutic agents may be delivered orally, either by oral gavage or in food and water. To assess the effectiveness of treatments, tumour burden will be monitored using imaging software, blood sampling, calliper measurements (tumour size) or at the end of the study, after the animals have been killed. The method of tumour monitoring will depend on the tumour model being used. During therapeutic interventions, it is possible that mice will be subjected to multiple procedures (up to 5 times/week). The duration of established cancer models is usually between 1-4 months; however, is also dependent on the cancer model. All animals will be killed humanely at the end of each experiment and mice may be killed at specific time points depending on the research question being addressed.

What are the expected impacts and/or adverse effects for the animals during your project?

General signs of ill health will be assessed throughout each study.

Injection of tumour cells, and the resulting tumour mass, may cause some pain and discomfort. However, in our experience tumour growth is extremely well tolerated. Hind limb paralysis/weakness is a common adverse event in some cancer models (e.g., multiple

myeloma), however, with daily monitoring this symptom is observed early, and animals are humanely killed at the first sign of hind limb paralysis/weakness. Potential, but unlikely adverse effects for tumour growth and metastasis could include weight loss, hunching, tremors or convulsions or altered breathing; however, we have not experienced any of these adverse events to date. All animals will be humanely killed if they display any sign of discomfort or deterioration of health.

The administration of therapeutic agents by different routes may also cause pain and discomfort; however, pain associated with therapeutic intervention will be short lived. No adverse events are expected as a result of therapeutic intervention, except short-lived discomfort at injectable sites, during oral gavage, or following repeated dosing. It is possible that mice may develop a fever after treatment with some biological agents, although to date, we have not experienced this in any of our studies. If this was an anticipated possible side effect, mice would be monitored carefully for signs of discomfort and humanely killed if symptoms did not subside within a couple of hours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All animals will be killed humanely at the end of the experiment and are expected to suffer no more than moderate suffering during the entire experiment. Moderate suffering is expected because of the cumulative effects of repeated procedures, although the treatment itself is unlikely to cause any adverse events except short-lived discomfort during treatment administration. Mice may also develop a fever after treatment with some biological agents, however, in our experience this is unlikely (<5% animals).

Moderate adverse events associated with injection of tumour cells, and the resulting tumour mass, may cause some pain and discomfort. However, in our experience tumour growth is extremely well tolerated. Hind limb paralysis/weakness is also a common adverse event in some cancer models (e.g., multiple myeloma). Potential, but unlikely (<5% animals) adverse effects for tumour growth and metastasis could include weight loss, hunching, tremors or convulsions or altered breathing; however, we have not experienced any of these adverse events to date.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of this project is to better understand the regulatory systems that exist within the tumour and identifying novel treatment strategies which make cancers more amenable to therapeutic intervention. Although we conduct many experiments in the laboratory to investigate the effectiveness of anti-cancer therapies, these models are not fully representative of what may happen in patients. This is because:

(i) cancer cells do not exist in isolation but co-exist alongside other cell populations which support cancer growth and development; and (ii) the response to therapy involves a complex set of interactions between different cell types and at different locations within the body. Therefore, to identify treatments that have the best chance of success when translated to cancer patients, they need to be tested in animals that model the full complexity of cancer environment and that have a fully functioning immune system.

Unfortunately, it is only possible to model simple interactions between different cell populations in the laboratory. However, this will be done as much as possible to replace and reduce animal experimentation.

Which non-animal alternatives did you consider for use in this project?

We use a range of non-animal alternatives to characterise cancer cells, the cancer microenvironment and the effectiveness of treatments. These include human cancer cell lines, human immune cells isolated from health donor blood, and cancer tissue and blood from cancer patients. We use these routinely in the laboratory, either cultured alone or in co-culture model systems to mimic cell-to-cell interactions that occur within the cancer environment. Animal work is only carried out when laboratory studies have been performed and data has confirmed that therapeutic strategies are effective against disease.

Why were they not suitable?

Cancer is a complex disease with multiple cell-to-cell interactions occurring within the tumour and unfortunately, these cannot be accurately recapitulated in the laboratory. Therefore, animal models are required to fully characterise the cancer environment and test the effectiveness of novel treatments.

Moreover, laboratory-based studies do not allow us to: 1) determine whether therapeutic agents can reach tumour sites after different routes of delivery, 2) establish the best route of delivery, 3) identify off-target effects to non-tumour tissue, or 4) optimise treatment schedules in a complete living system capable of inactivating and/or clearing drugs from the body. Taking this into account, we rely on data obtained from well-designed animal experiments to determine the true efficacy of drugs before translation into patients.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This animal number has been estimated based on current and planned research activity. Protocols are based on previous work where statistical differences in tumour burden or survival have been demonstrated. Typically, group sizes require 6-10 animals (dependent on mouse model/therapy) to provide sufficient power to detect statistically significant differences. In vivo passaging of cells is required for some tumour models, where this is required animal numbers are low and determined depending on the downstream experiment; typically, tumour cells obtained from one animal is enough to seed tumours in 4 recipient animals.

Where possible, we will use imaging and blood sampling to monitor tumour burden longitudinally in the same animal to avoid unnecessary killing. This will reduce the number of animals used.

As part of good laboratory practice, we will write standardised protocols for each experiment including the study objective(s), experimental plan (animal numbers/groups and schedules) and methods that will be used for downstream analyses of results. While the overall numbers of animals may appear large, 2500 animals over 5 years equates to 500 animals/year to cover a range of different research projects, carried out by multiple staff. The estimated use for protocol 2 and 3 (therapeutic intervention protocols) is 1000 (protocol 2) and 1300 (protocol 3) animals over 5 years. An additional 200 animals are expected to be required for protocol 1 (in vivo passage of cells). In total, this is the equivalent of 500 animals/year for the project.

A typical study would have a minimum of 6-10 animals/treatment group, simply testing one agent vs controls would be 12-20 animals for one small experiment, and 3 biological repeats of the same experiment would require 36-60 animals. Moreover, more complex testing of combination therapies, which requires testing of single agents as well as the drug combination, means that these numbers rise rapidly. The maximum estimated number of animals required for this project were therefore calculated are based on the last 5 years' experience of working with different cancer models, alongside current and predicted research activity.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Protocols are based on previous studies where efficient statistical differences have been demonstrated. In therapeutic studies (protocol 2 and 3) typically 6-10 animals are required

per treatment group (dependent on mouse model) to provide at least 80% power to detect differences using analysis of variance. Routine passaging of cells (protocol 1) will be decided based upon the number of animals required to generate sufficient cells for subsequent downstream studies. Normally tumour cells from one animal will be passaged into 4 recipients, with some cells remaining for the generation of frozen stocks to be used at a later date.

We will continue to re-evaluate animal numbers in line with new developments and technology. In particular, we would like to improve in vivo tumour imaging which will enable us to carry out longitudinal experiments (with repeated measurements) in the same animal, rather than having to kill cohorts to obtain the same endpoints (i.e. tumour burden) ex vivo. Advances in these areas will improve experimental design and group randomisation prior to therapeutic intervention

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continually review animal numbers throughout the course of this project license and try to establish more sensitive methods for monitoring tumour burden (e.g. imaging technology). For each experiment, we will ensure that tissue from each animal is fully utilised, this will allow us to thoroughly assess biological changes and maximise the output from each study. For example, we will endeavour to share tissue that is not needed for our study with other researchers, so all tissue is fully utilised.

For new studies, where data on therapeutic efficacy is not available, pilot studies and power calculations will be carried out to estimate the size of the likely therapeutic benefit before group sizes are finalised. Statistical advice will also be sought throughout the programme to take into account any new developments and reduce animal numbers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mouse tumour models

Mouse models will be used because these are the lowest form of mammal recognised as relevant for human cancer. Mice do not develop spontaneous cancer at a rate compatible

with experimentation, therefore tumour cells will be implanted into the mice. In general, we will use two types of mouse models to study cancer and the effects of new or existing cancer therapies. The first model uses mouse tumours implanted into mice with a fully functioning immune system. This is important as the therapeutic agents we test are act on immune cells (e.g. termed immunotherapies) and harness the immune system for their anti-cancer effects. Tumour models will depend on the study but will encompass models of both haematological malignancies and solid malignancies. A second model uses human tumour cells implanted into mice that lack part of their immune system or mice that have been engineered to develop parts of the human immune system. These models will be used less frequently but are important when mouse models of cancer do not closely resemble the characteristics of human disease. As tumours grow in each of these models, the health of all animals will be closely monitored to avoid pain and discomfort associated with tumour growth. If necessary, animals will be humanely killed.

Methods

Throughout this project, we will use practices that minimise stress, harm and pain to animals. For example, we will only use single-use needles for injections to avoid pain from dulled needles. For tumour cell injection we will do this via the tail vein (for models of haematological malignancies), intraperitoneally to model ovarian cancer or subcutaneously for other cancer types (e.g. colorectal cancer). After tumour cell implantation, we will monitor tumour burden using various methods depending on the tumour model, these include bioluminescence (IVIS) imaging, blood sampling, assessment of animal grith/weight, calliper measurements, and/or monitoring of disease-related symptoms. These studies will be done with and without therapeutic intervention.

All procedures are mild, and it is not anticipated these will have adverse effects. However, mice will be monitored daily for symptoms of disease or adverse events associated with treatment. Our work is based on the humane treatment of animals and all work will be carried out by fully trained staff to ensure the highest standards. Discomfort will be limited to unavoidable procedures that are required to conduct valid research. If at any time any animal is showing signs of ill health or continued distress they will be killed humanely.

Why can’t you use animals that are less sentient?

A whole organism is required to establish the effectiveness of novel anti-cancer treatments. As such, mouse models are extensively used in the study of cancer, cancer immunology and to test cancer immunotherapies. There is a wealth of information available on mouse models and they are reliable and reproducible models of the disease. Mice are also very similar to humans genetically, and the process of cancer development is conserved between mouse and humans. Mice that lack a fully competent immune system also allow the growth of human tumours and also allow us to study human disease in a living organism.

Less-sentient species (e.g., zebrafish) can be used to study tumour development; however, restricted tumour size means that are unlikely to develop the full repertoire of immune evasion strategies that prevent the immune system from eradicating the tumour. Thus, this makes it difficult to establish the true effectiveness of novel immunotherapy strategies. For our studies, which are aimed at examining the efficacy of treatments against primary tumours and/or metastatic disease, mice are the most reliable to model tumour progression and treatment delivery.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

General signs of ill health and discomfort will be assessed using the principles of the grimace scale for mice. Moreover, mice will be monitored regularly for other signs of discomfort, including any changes in behaviour, weight loss and movement. We will also attempt to identify earlier endpoints for our studies, to minimise harm and potential discomfort. To minimise harm to animals, we will strive to use refined mouse models of cancer which identify earlier endpoints that do not rely on the animals showing signs of being unwell, suffering and pain to mice. Where possible, we will use specialised non-invasive imaging techniques (carried out under anaesthesia) that can detect the disease at much earlier stages. We will make sure that each cancer model is reliable and reproducible, this will also allow earlier endpoints to be used. For example, increased monitoring around the expected study endpoints (e.g., via imaging, blood sampling, calliper measurements or detailed visual inspection for early disease- related symptoms) will allow mice to be humanely killed before significant disease-related symptoms develop. Some of these refinements will also allow reduce the number of mice required and tumour burden and biological readouts can be observed and monitored in living animals.

Only animals that are in good health will be use and animals which lack a fully functioning immune system will be housed in ventilated cages to minimise their risk of infection. Any procedures that could lead to pain or discomfort will be performed with anaesthesia or appropriate pain relief. All animals will be regularly monitored for possible adverse effects that may occur in response to the tumour growth, procedures or treatments. If required, we will seek advice from the Named Veterinary Surgeon (NVS) and Named Animal Care & Welfare Officer (NACWO) (e.g. for administration of antibiotics if an infection is suspected). Small pilot studies will be used to identify optimal treatment regimens including drug dosing, timing of therapeutic intervention and route of administration before proceeding to larger scale studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Procedures for establishment and monitoring of tumour growth, metastasis and animal welfare will follow best practice guidelines (e.g. NCRI Guidelines for the welfare and use of animals in cancer research, Workman et al. 2010 Br. J. Cancer). We will also consult the

NC3Rs website for information on the 3Rs. Published guidelines (Guidance on the Operation of the Animals (Scientific Procedures) Act 1886) which highlights recommended volumes for blood sampling and number of procedures will be followed.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I regularly receive NC3Rs online resources and updates and am a member of our local HO licence holder committee which meets regularly to discuss animal welfare and important developments. I also attend local, national and international conferences and read peer-reviewed literature to remain up to date with new advances. Importantly, animal experimentation will not be carried out until we have generated a significant amount of in vitro data demonstrating the effectiveness of novel therapies against human disease, and the appropriateness of selected mouse models.

31. Investigating the Role Of Platelets in Haemostasis, Thrombosis and Immune Response

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Platelets, Immune cells, inflammation, Haemostasis, Cancer

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the roles of blood cells, mainly platelets, in stopping bleed, heart disease and regulation of the immune system.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Identifying the role of newly identified proteins/receptors in the regulation of platelet function contribute to research data that can be used in the development of effective new therapies, such as drugs that prevent strokes or excessive bleeding, and/or repurposing of clinically approved drugs that may be beneficial in other disease conditions.

The assessment of platelet function from mice whose proteins of interest have either been deleted or mutated provides a practical understanding of their involvement in diseases. Furthermore, a particular protein that may have a minor or substantial effect in isolated cells may have a greater or lesser role in the complex in the body environment where other factors may influence its actions. Thus, the true potential contribution of key platelet proteins/receptors needs to be assessed in a physiological context to understand their real-world function.

What outputs do you think you will see at the end of this project?

Our proposed programme aims to establish novel and previously unexplored (and underappreciated) roles for platelets and platelet proteins/receptors in the regulation and modulation of haemostasis, thrombosis, and the immune system, thus contributing to knowledge of genes and proteins that are candidates for cardiovascular disease. As such, our research is of relevance to and has the potential to have impact upon (i) clinical research scientists, who will use our data to screen patients for biomarkers and to inform and guide future clinical studies/trials; (ii) pharmaceutical industry researchers who could develop our disease models and transgenic animals for drug screens; (iii) Appropriate data/findings will be shared via high impact, peer-reviewed publications and we anticipate that data from this project will initiate several follow-on studies. The applicant is also very active with public outreach activities and on Twitter and it is expected that our data/findings will be of interest to online and print scientific magazines as well as charities (e.g., the British Heart Foundation and the Medical Research Council).

Our studies of the mechanisms of CVD have led us to address links between oxidised low-density lipoproteins and platelet dysfunction. Our current area of interest and expertise lies in crosstalk between the immune and cardiovascular system. Understanding these intricate systems is complex. For our research, we are using mice to make models for disease (such as sepsis and dyslipidaemia) we see in humans to examine the role(s) platelets play in the development of these disease. We are using tests to identify new drug targets and find new genes/proteins that lead to/aggravate disease symptoms. We hope that we will identify biological markers and molecules that may lead to the identification of drugs that are more effective for treatment in humans.

Who or what will benefit from these outputs, and how?

Pathological thrombosis is a major component of the pathology that underlies cardiovascular diseases (CVD) and other chronic diseases. In the UK, CVD accounts for 28% of annual deaths and is the greatest cause of mortality, killing approximately 161 000 people in 2012 (www.heartstats.org - British Heart Foundation). Furthermore, these

numbers do not take into account the thrombosis related deaths associated with diseases such as cancer and diabetes. Thus, thrombotic disease inflicts significant health and financial burden on the UK. More recently, platelets are understood to modulate the immune response to inflammation and cancer, but the mechanisms remain unclear.

In the short term, the data generated in this project will establish the role of platelet proteins/receptors in CVDs and immune response to infection, thus providing further opportunities to drive academic excellence. In the absence of key data on the role of platelet receptors, such as PD-L1, it is currently not possible to assess the effect of anti-PD-L1 drugs in the prism of platelets. Therefore, using a platelet-specific PD-L1 KO mouse model will clearly define the role of this receptor in thrombosis and inflammation. This will inform the direction of future research that we disseminate to scientists and medical doctors through conference presentations, and publication of the data in scientific journals using the ARRIVE guidelines on animal experiment reporting. As standard with good academic practice all models developed in our laboratory would be shared with the wider scientific community, both directly within our institute and wider afield. Should our work lead to refinements in laboratory practice for example as improved surgical, imaging, or experimental procedures these would be made available for other researchers to utilise.

On the long term, based on the results, selective drugs targeting platelets can be administrated, in particular drugs available on the market that can be re purposed to use in patients with CVDs and/or sepsis. Identifying a target that can reduce thrombosis and inflammation is crucial to justify a clinical trial that can reduce pain in patients, decrease organ damage and increase survival in these patients. There are also potential advantages for the pharmaceutical industry, which remain highly interested in developing agents to control unwanted platelet activation.

Our work programme, therefore, not only facilitates correct understanding of CVD and inflammation, but may also provide an avenue for effective treatment.

How will you look to maximise the outputs of this work?

We will publicise our findings to the wider scientific community, industry and the public through annual conference presentations, publications and other media.

Our work is presented on a regular basis within our institute, through research seminars, and often disseminated through academic conferences. These are used to discuss the work in progress and provides a forum with which we can discuss experimental issues (including animal welfare and phenotypes) informally with other experts in order to refine techniques and help develop best practice. The publication of our work is another major form of dissemination, where we report the findings in a more formal manner. In our recent work, we used a similar approach to that outlined in this application to identify a new mechanism of thrombosis.

We aim to publish at least two high impact publications, which build on our previous work. Upon publishing our research results in scientific journals, we will prepare press releases in collaboration with the press offices of the University. This transparency of approach to sharing data will maximise our outputs, ensure that our research activities are complementary rather than competitive and that the field moves forward as quickly as possible.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We plan to use adult mice as a model for human platelet function. Well-established and standardised tests allow our data to be compared with a large body of scientific literature. While there are subtle differences between mouse and human platelets, in most cases mice model normal and diseased processes in humans well. Through the use of genetically modified mouse models, this project also allows us to overcome the limitations that are caused by an inability to perform molecular biology on platelets, due to lack of nucleus.

Typically, what will be done to an animal used in your project?

In this project, blood will be taken from adult mice, some of which are genetically modified, and blood cells isolated to be examined in the laboratory. Some mice will be subjected to haemostatic or thrombotic in vivo assays under general anaesthesia. Under some protocols, adult mice might be fed a special high fat diet that mimics Western-styled food, for up to 4 months, to examine the effect of such diet on blood cells. In other protocols, adult mice might be injected with agent(s) to provoke an immune response to investigate the complex relationship between platelets and the immune system.

What are the expected impacts and/or adverse effects for the animals during your project?

None of the mouse strains that we currently hold or are interested in bringing in under our project licence have an overt or adverse phenotype under normal conditions. However, there are three areas where adverse effects may be anticipated.

1. Dietary manipulation - on rare occasions some mice find high-calorie diets unpalatable and begin to lose weight. Mice will be monitored daily for their general well-being and weighed on a weekly basis. Should an animal show signs of distress, it will be

removed from the study and returned to a normal diet. If the distress continues, the animal will be culled by a schedule 1 procedure.

2. Administration of substance - There could also be potential impacts of administration of substances designed to influence the recruitment and/or function of circulating platelets and/or immune cells. Substances will be administered where dosing and potential toxicity data are available in the mouse or similar species. If adverse effects are suspected or reached the maximum allowed score (protocol 5), the animals will be withdrawn from the study. If the adverse reactions persist, the animals will be killed by a Schedule 1 or other method stated in the protocol immediately. As data is collected, we will review the protocol to ensure that the severity limits are appropriate.

3. Recovery during anaesthesia - The severity of this procedure will be controlled through the use and careful monitoring of general anaesthesia throughout. There is a possibility the animal recovers prematurely from anaesthesia, although this would be a relatively rare occurrence. To prevent premature recovery from anaesthesia there will be continuous monitoring of the depth of anaesthesia by testing the limb flexion/withdrawal reflex and/or the corneal reflex, which will be supported by continual monitoring of heart rate and body temperature. Should there be suspicion that the animal is recovering prematurely additional anaesthetic will be administered by inhalation. However, it is noteworthy that this has not occurred during the procedures performed in the last five years and therefore we do not anticipate this to be a common occurrence.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity of our procedures is categorised as non-recovery, mild or moderate. The majority of our procedures (>80%) are carried out under non-recovery, while the remaining procedures (<20%) will be either mild or moderate.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although we are committed to using non-animal methods wherever possible, it is difficult to mimic integrated physiological processes and behaviour in in vitro systems, in addition to ethical limits on human experiments. Often using animals is the only means of achieving the objectives of experiments. Moreover, the study of blood platelets is dictated by three limiting factors.

1. We minimise the use of animals by using platelets from human volunteers. However, the experimental approaches that we can use with human platelets are limited. This is because platelets do not contain genetic material (i.e. DNA). This means that they cannot be grown in culture and cannot be modified through standard genetic techniques used in other cell types. Mouse gene knockouts provide a powerful approach to understand the role of a specific gene in platelet function. This cannot be replicated with human platelets.

2. Complete replacement of immune-challenged animal models is difficult. The conglomerate of immune responses associated with infection or cancer is too complicated to model in cell culture systems. This is coupled with the inability to culture platelets ex vivo.

Therefore, the use of genetically altered knockout mice to identify and delineate the pathways involved in platelet activation is required. Our laboratory is committed to performing work with animals only when the potential biomedical advances warrant this. We are interested in developing new approaches that would reduce the use or generation of genetically altered mice to study platelet function, and we are closely monitoring studies in other laboratories that have begun to examine ways to produce genetically modified platelets (using siRNA technology) in vitro from bone marrow culture. Should this technology become available, we will look to adapting it for our own studies.

Which non-animal alternatives did you consider for use in this project? Pharmacological inhibitors that target specific proteins/receptors using human platelets. Why were they not suitable?

In vitro studies using human platelets and pharmacological inhibitors are showing a great deal of promise to study platelet function. However, this model remains imperfect and fail to capture the complexities of human body, which involve complex interactions between many different cell types, and in many cases communication between the vascular and immune system

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In the types of studies we perform, the anticipated response is usually “all or nothing", that is the genetically modified mice fail to respond to stimuli when compared to the wild type. Therefore, the numbers for in vitro studies of our programme are calculated based on the number of samples we can prepare per mouse. For example, the blood from each animal provides approximately 1 ml of washed platelets which is sufficient to prepare for four samples. With this knowledge, we can extrapolate the appropriate number of mice required by determining how many samples are required for each series of experiments within our project. Under these controlled conditions we aim to use approximately 6 mice per strain per assay (and 6 control animals) since platelet responses are generally very reproducible.

In general, we use between 10 and 15 assays during our functional evaluation.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our experimental design is driven by studies performed on human platelets, which act as a guide to the potential importance of key proteins in the function of platelets. Careful analysis of the data emerging from these experiments will determine the need for experiments in animals. It is noteworthy that the experiments performed under this licence are discovery science and so very little information is available to guide our calculation of numbers. However, based on your experience of performing similar studies in mice in the past, we can reasonably estimate the lowest number of animals to gain meaningful results and whether extensive experiments using multiple in-depth assays are required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have a number of strategies for optimising animal numbers.

1. The key step is the generation of pilot data from in vitro functional assays. Lack of phenotype in these assays (usually between 10 and 15 assays) prevents mice from being used in experiments that require dietary manipulation and reduces the breeding of mice.

2. All other tissues (e.g; muscles) from mice sacrificed under any of the protocols are available for other researchers within our institute.

3. Tissue (e.g; kidneys, heart, liver) and whole blood analysis will be performed following schedule one of our immune-challenged animal model (protocol 5). This will greatly reduce the number of animals sacrificed and provide more meaningful data.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this project, we will use mice (wildtype and genetically modified) mainly bred on a C57/BL6 background. Initial animal health will be verified prior to importation to ensure the welfare of the animals. No experiments are performed on newly imported animals for at least 7 days. Mice will be bred and maintained in our facility at the University of Hull. Our breeding facility is meticulously maintained by qualified staff that implements rigorous health checks on mouse colonies, minimizing the risk of infection. Experiments will be performed to investigate the role of blood cells in haemostasis and immune response. Currently, the mouse haemostatic and immune systems are considered a strong model for that found in humans and has the advantage of genetic manipulation. However, there are a number of areas where we have refined our programme of work.

Protocol 1. We have now established ear punching for biopsy collection. We use ear punch for identification purposes and the resulting sample is used for genotyping. This is kept to a minimum if modified strains we use are bred as homozygous coupling meaning that only the initial parentage requires identification and genotyping and beyond this, it is performed in a random fashion. Hair samples might be used for genotyping should we find the data reliable and accurate. Our genotyping data will be verified by determining the absence of the deleted protein(s) from platelets using Immunoblotting. Animals are kept under supervision following biopsy until the bleeding has ceased. If bleeding does not cease within 15 minutes without intervention, or if the application of pressure to the wound does not cease bleeding, the animal will be culled by an approved schedule 1 method. Should excessive bleeding occur during ear punching for biopsy, the affected strain of mice would not be subjected to tail bleeding assays (protocol 2) to avoid any excess suffering.

Protocol 2. This procedure is performed under terminal anaesthesia as a non-recovery protocol in order to avoid subjecting the mice to a painful procedure. To avoid excessive bleeding and stress for the animal, we restricted the procedure to 20 minutes or 15% blood loss- based on a circulating blood volume of 1.5-2.5mL (whichever happens first). At which point the experiment is stopped and the animal is humanely culled (Schedule 1 method). We will also adopt other methods to quantify bleeding time measurement (e.g. OD and HB measurement following bleeding- time measurement). Cross- validation of our data will result in less variation and thus fewer animals will be used.

Protocol 3. As part of refining our procedures and reducing animal usage, we have moved over to using tail vein injections for the delivery of antibodies for the labelling of platelets. This refinement is much quicker and less invasive than other methods (give examples), minimizing any pain the animal might encounter. Moreover, this allows us to use both carotid vessels for thrombosis assays and will therefore reduce the number of animals required.

Advice will be sought from the NVS and NACWO about new advances and developments in surgery procedures and/or anaesthesia that might lessen the pain for mice. In all cases, at the end of Protocol 2 and 3 animals will be swiftly and humanely killed to minimize the severity of the procedure.

Protocol 4. High-fat diets can induce severe phenotypes in genetically modified mice designed to provoke extreme hyperlipidaemia. We have avoided specifically these “diet-sensitive” mice and used a very short-term feeding regime. This approach, if successful, will allow us to develop models of disease that more accurately represent the human disease condition without having to keep mice under long feeding regimes in which serious health issues are known to occur.

Protocol 5. For our model, we will use established protocols that invoke immune challenge in mice. Animals will be routinely monitored and checked against a comprehensive list of clinical signs and symptoms. Animals that cross our severity threshold will be culled immediately using a Schedule 1 protocol. To minimise unnecessary suffering, strains that are known to tolerate immune-challenge will be used. Clinically relevant signs will be used to assess animals as an endpoint instead of death.

Sublethal doses of LPS will be used (see protocol 5).

Why can’t you use animals that are less sentient?

Less sentient animals such as fish or frogs are not appropriate for our research as these differ in their haemostatic and immune response.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The major refinement to our protocols is in the area of in vivo thrombus formation. We have now moved away from cannulating the carotid artery to inject antibodies to label cells (platelets), and we are now using tail vein injection instead. The success rate of cannulating the tail vein is significantly higher and more reproducible, thereby reducing the number of mice required for experiments.

Furthermore, since this procedure is performed under terminal anaesthesia, it minimises suffering in the mice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Major guidance through the project is taken from the NC3Rs, whose website (hubs and microsites) provides a vast array of resources on the general principles underlying the experiments highlighted in this project; this includes anaesthesia, breeding strategy and numbers, and experimental design.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will use regular literature searching and the NC3Rs website to gather up to date information about advances in animal research. This information will be disseminated to anyone involved with this licence including the project licence holder (PPLH), personal licence holders working under the licence (PILs) and named animal care and welfare officers (NACWOs) and implemented where appropriate.

32. Cardiovascular Disease: Underlying Mechanisms and Detection

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

atherosclerosis, blood flow, heart failure, arterial wall permeability

Animal types	Life stages
Rabbits	juvenile, adult
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The major aim of this work is to increase our understanding of atherosclerosis, a disease characterised by the accumulation of fat (especially cholesterol) within the arterial wall. Subsidiary aims are to improve ultrasound-based methods for measuring details of blood flow near the arterial wall, since the development of the disease depends on such details, and to improve the similar, ultrasound-based methods for measuring pulse waves in arteries, since these can be used to assess risk factors for the disease and also consequences of the disease such as heart failure.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Atherosclerosis is the main underlying cause of heart attacks, strokes and peripheral artery disease and is thereby one of the largest causes of morbidity and mortality in Western countries, and increasingly important in developing countries, despite the availability of drugs that lower classical risk factors such as high blood pressure and high cholesterol levels in blood. We need more information about how the disease develops in order to design new ways of intervening in the process. The information we need includes details of blood flow, and we are developing new techniques for their measurement. These techniques have other uses, including in diagnostics. Concerning the simple, low-cost detection of heart failure, there is a clear unmet clinical need for this. For example, 75% of patients with heart failure in the UK are diagnosed as a result of an acute hospital admission, even though 50% had been to their GP with symptoms in the previous 5 years.

What outputs do you think you will see at the end of this project?

If all the projects receive funding , outputs would be: (i) identification of new pathways that control the rate at which cholesterol enters the arterial wall from blood, (ii) identification of possible points or molecules that could be used to modify such pathways, (iii) determination of whether the data obtained in the aorta are also relevant to the more clinically relevant coronary arteries, (iv) algorithms for measuring the stresses imposed on the arterial wall by the flow of blood, and for measuring properties of pulse waves in arteries, by ultrasound, and (v) data on such stresses and pulse waves. We aim to publish all our data as full papers in refereed scientific journals.

Who or what will benefit from these outputs, and how?

In the short-term, our data will benefit other research groups investigating how arterial disease develops, or attempting to measure arterial blood flow or pulse waves. In the long-term, the pathways may lead to new therapies for reducing atherosclerosis, and the methods may lead to new techniques for diagnosing disease.

How will you look to maximise the outputs of this work?

Our work is highly collaborative, including other principle investigators in this Department with expertise in cell signalling or ultrasound, and with collaborators in Aeronautics who are experts in flow. Knowledge is disseminated through publication in scientific papers (I have published >100) and, more rapidly, by attending conferences. Unsuccessful approaches are discussed in our papers.

Species and numbers of animals expected to be used

- Mice: 150
- Rabbits: 150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

When we examine fundamental processes that should apply to the same cell type in any mammalian species, then we use mice. For example, mice are used when examining the fundamentals of the transport of cholesterol across the cells that line the inner surface of our vessels, forming a barrier between blood and the underlying wall. (Cholesterol and other fats do not dissolve well in blood, and they are therefore transported bound to proteins, as part of particles call lipoproteins.) We use rabbits when we examine specific pathways the might form a link between blood flow characteristics and arterial disease. That is because the characteristics of blood flow scale with body weight; stresses in rabbits are much closer to the stresses occurring in people and indeed we have validated that they are sufficiently similar for this purpose. Mice are not. Finally, we use rabbits for the ultrasound methods simply because their larger size gives us better resolution compared to the dimensions of the arteries.

Typically, what will be done to an animal used in your project?

There is a range of procedures. (i) In the simplest, an animal might receive an intravenous injection of a blood thinning agent so that arterial tissue can be harvested after death without blood clots forming.

(ii) For the measurement of flow and pulse waves, the animal would be terminally anaesthetised and then ventilated, allowing imaging to take place without animal movement. Drugs are sometimes given under terminal anaesthesia (i.e. anaesthesia without recovery) to modify the properties being imaged.

(iii) A very common procedure is the give an injection into a vein of a naturally occurring molecule with a dye attached to it. This is allowed to circulate for times up to a few hours, and then the animal is given the blood thinner, anaesthetised, and its blood vessels chemically "fixed" at the same time that the animal is killed, so that we can measure how much of the tracer was transported into the wall. ("Tracer" means a small fraction of molecules of one type that are labelled so that they can be followed

- for example by attaching a dye to some of the lipoproteins mentioned in the preceding section.) In some of these experiments, the animal might additionally be given a non-harmful drug orally or intravenously in an attempt to modify movement of the tracer. (iv)

Less commonly, vessels are perfused with tracers, and sometimes drugs, after cannulation in situ under terminal anaesthesia. (v) The most complex experiments involve feeding an animal a cholesterol-enhanced diet for several weeks in order to induce mild atherosclerosis. Again, drugs might also be given to modify the disease, either orally or through the implantation of small pumps under the skin. The latter involves recovery surgery, but the pumps (which resemble capsules) are small and recovery is rapid. Again, drugs are non-harmful.

What are the expected impacts and/or adverse effects for the animals during your project?

Almost no adverse effect is expected from any of these procedures since they involve only oral or intravenous administration of non-harmful substances, procedures under terminal anaesthesia, or administering diets that induce only the early stage of arterial disease, withoutu symptoms. Mature rabbits are sometimes reluctant to switch diets and this may result in weight loss - methods for stimulating appetite will be used in such cases. Administering drugs during the induction of disease involves recovery surgery if the dugs require the insertion of pumps under the skin - this is a standard procedure and adverse effects can be alleviated by simple pain-reduction routines.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Rabbits 27% mild, 40% moderate, 33% non-recovery

Mice 47% mild, 20% moderate, 33% non-recovery

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Arterial disease and the processes on which it depends, including subtle features of blood flow or transport properties of the arterial wall, can only be conducted in vivo; the circulation, and indeed the fat metabolism of the animal, need to be intact. The same applies for determining whether imaging methods can be useful for measuring blood flow

or arterial waves in vivo - blood needs to flow, pulse waves need to propagate, and overlying tissue needs to move and generate noise for this to be assessed.

Which non-animal alternatives did you consider for use in this project?

The animal-based work is supported by other types of work. Cell culture is used to study mechanisms at the molecular and cellular level. For example, FSTL1 was indentified as a target through such experiments. We have applied for funding to continue such work e.g. to identify other targets. We use in silico methods (computational fluid dynamics) to simulate blood flow, but such simulations require acurately defined inflow, outflow and geometric "boundary conditions" that can only be obtained in vivo. We are already testing early versions of our heart failure diagnostic methods in patients, but animal work is required to further refine the methods and to compare the results with "gold standard" data obtained using invasive techniques such as arterial catheterisation. Finally, we aim to submit a grant proposal for a study examining aortic geometry and disease location in cadaveric human specimens with abnormal spinal curvature, such as scoliosis; this would be expected alter the pattern of blood flow (which we can compute) and, if our theory is correct, disease location should change in a corresponding fashion.

Thus we have considered using computers to simulate blood flow, measuring permeability of the cells lining the arterial wall in culture, and testing imaging methods in models such as latex tubes with artifical blood flowing through them. These are all excellent methods and we routinely and extensively employ them in our work but they cannot fully replace the use of animals, especially in studies of the effects of mechanical stresses and drugs on transport and disease, for which an intact organisms is required. The early stages of the disease, which is the focus of our research on arterial disease, are unfortunately too mild to be imaged non-invasively in people, e.g. by MRI scans.

Why were they not suitable?

These systems only give preliminary answers; the insights obtained from such methods are essentially hypotheses that need to be tested in vivo because the models do not completely replicate the complexities of intact physiological systems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have submitted a grant application to study arterial disease which proposed the use of 62 rabbits. This typifies the numbers used per grant. We have several more proposals in preparation but it is not possible to say how many will get funded. As an estimate, therefore, 50 animals of each species have been included in each protocol.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Effects of each drug tend to be tested separately, due to the nature of the funding stream, and are therefore tested simply as experimental versus control. (Controls will be shared for two or more drug groups when this is feasible.) The size of each group is calculated according to the minimum useful size of the effect, the variation between animals and the statistical power that is required. (Effect sizes are taken as 20% since smaller effects would be hard to detect in clinical trials if translated to human subjects.) In some cases this produces very low numbers (e.g. 2 or 3), which causes great difficulty when attempting to publish the work; in such cases we use the minimum number that is acceptable to journal editors and referees.

The ultrasound trials are exploratory and cannot be quantified in this way: the minimum number is used to develop the technologies. No further animals would be used when, say, the device produces measurements with sufficient discriminatory power for the results to be useful, or when this has not been achieved but there are no practicable methods for further improvement.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Tissue is stored for future use (e.g. for staining of molecules to identify future drug targets). Tissue from other organs is shared with groups within our Department. Pilot studies are conducted when administering drugs of unknown efficacy.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Models are: mice and rabbits, vessels cannulated and perfused in rabbits under terminal anaesthesia, genetically modified mice that develop arterial disease, and rabbits fed a

cholesterol-enhanced diet. All drugs administered to conscious animals are non-harmful and all diets are administered for durations that induce only asymptomatic disease ("fatty streaks"). There is very slight pain due to intravenous injections. Pain arising from an aseptic implantation of osmotic minipumps under the skin that is effectively controlled by the administration of suitable pain relief as recommended by the establishment vet.

Why can't you use animals that are less sentient?

Most procedures do not involve any pain or harm, or are conducted under terminal anaesthesia, so these considerations do not apply. Animals have to be sufficiently old for diets that induce arterial disease to be administered. Species are the least sentient that are known to develop atherosclerosis with the spatial distribution that we require: only rabbits are known to show the age related distribution of lesions around branches that are seen in human aortas. For studies where these distributions are not important (e.g. when investigating fundamentals of transport across the layer of cells lining blood vessels), mice will be used. We would not wish to use cold-blooded species since their lower temperatures will affect the physics of transport. For example, diffusion of molecules through the gaps between cells is highly dependent on temperature.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals undergoing implantation of pumps under the skin are given pre-emptive analgesia. For animals that are administered diets to induce arterial disease, dietary supplements are increased gradually. If there are signs of adverse effects despite these precautions, alleviation will be provided,

e.g. temporary return to a normal diet, or an increase in analgesics after osmotic minipump insertion, and appropriate humane end points applied if necessary.

During the course of regulated procedures, animals may be transferred to other sites within the primary Licensed Establishment. Such transfers will only be undertaken where scientifically justified (e.g. where tissues or cadavers will not suffice); only if the animals have been inspected by a competent person and deemed to be in a suitable condition to be transferred; the transfer is conducted in a manner to minimise distress and there is no reason to believe the animals will suffer as a consequence of the transfer. The Home Office will be promptly notified of any unexpected welfare problems associated with transferring animals. In order to ensure that any additional harms to the animals associated with transport, and any potential effects on the quality of the science are both minimised, the following post-procedural recovery and acclimatization guidelines will apply. Normally, medically and surgically prepared animals will be allowed a minimum procedural or post-operative recovery period of 7 days prior to transfer and all animals will be allowed a minimum acclimatisation period of 7 days at the destination campus, prior to any subsequent use in regulated procedures. These periods may be varied, subject to the prior written approval of the NVS, in cases where there is a clear justification for this.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will consult guidelines of the Laboratory Animal Science Association for dosing volumes and resources of the National Centre for the Replacement, Reduction and Refinement of Animals in Research for techniques. Since many procedures are conducted under anaesthesia, the correct administration of anaesthesia is critical. For this, we follow standard textbooks and information for vets/veterinary nurses in small animal anaestheia.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through reading of the scientific literature and through information and courses promoted by the institution, and by signing up to the NC3Rs newsletter. Note also that I have lectured to undergraduates on animal models and on animal welfare whilst at a previous University.

33. Genetic and Environmental Regulation of Adiposity

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Diet, Adipose tissue, Genetics, Zebrafish

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to utilise zebrafish as a model organism to better understand how genetics and environment interact to regulate fat tissue traits such as (i) body fat levels (such as in obesity), (ii) distribution of fat accumulation in different areas of the body and (iii) function of the body fat in the context of obesity and associated disease. This work will help us to understand genetic predictors of disease risk, how they interact with the environment (e.g., diet) and identify new potential therapeutic targets to treat obesity-related disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The majority of adults in the UK are either overweight or obese (67% of men, 60% of women, NHS statistics 2021). Obesity is a primary risk factor for many 'cardiometabolic' diseases including cardiovascular disease and diabetes, along with cancer and neurodegeneration. However, we currently do not have sufficient knowledge on how the genetic makeup (or genotype) of an individual can cause obesity and how an individual's genotype interacts with an obesogenic environment characterised by high-calorie diets and low levels of physical activity. This limitation hinders our ability to predict obesity-related disease and design new therapeutics to alleviate obesity-related disorders. Obesity is characterised by increased body fat (adipose), and it is the quantity, composition and regional distribution of body fat which are primary drivers of disease risk in obesity. Body fat levels, distribution and composition are heritable and have a strong genetic basis. Therefore, it is important to identify and understand the genetic factors that regulate body fat levels and distribution. The zebrafish model system has many similarities to human and can be used to model the cellular, molecular and genetic mechanisms underlying obesity-related diseases in humans. Importantly, using zebrafish has several experimental advantages for studying body fat and obesity-related diseases. Undertaking this work will provide new understanding of genetic risk for obesity-related diseases and will therefore benefit a large section of the general public.

What outputs do you think you will see at the end of this project?

This project will teach us how genetics can contribute to obesity and cause obesity-related disease. We will identify specific genetic mutations (which we are born with and inherit from our parents) which

(i) influence an individual's propensity to become obese, (ii) influence how body fat is regionally distributed and (iii) how body fat functions. Collectively, these determine an individual's susceptibility to suffering from obesity-related disease. In particular, we will understand how body fat responds to an obesogenic environment (in the form of high-calorie diets and increased eating). For example, we will identify if a specific gene mutation causes more belly or leg fat to expand in obesity. It is these differences in body fat distribution which can exacerbate obesity-related disease. We will publish our findings and experimental insights in scientific journals which will be open access to the public. We will present our research findings at scientific conferences to disseminate information to the scientific community, and we will also undertake public engagement activities to disseminate our research to the general public. We will work with academic and industrial collaborators to help progress obesity-related drug development projects. Our long-term

goal is to understand obesity and associated disease and identify new treatments to predict and ameliorate obesity-related disease.

Who or what will benefit from these outputs, and how?

This project will benefit numerous groups including, the scientific community who will benefit by obtaining new information relevant to obesity and obesity-related disease which will augment their own research findings. This knowledge exchange will accelerate the development of obesity-related treatments. Pharmaceutical companies will also benefit in a similar way to this knowledge exchange which will further accelerate the development of obesity-related treatments. This project will utilize genetic data from humans. As such, our results will be directly relevant for patients and the wider population, and also provide new therapeutic targets to modulate disease risk. With the dawn of personalized medicine, the identification of important disease-associated genetic elements by this project will enable prediction of obesity-related disease risk in patients prior to actual disease onset.

The general public will also benefit from knowledge garnered during this project which will contribute to better understanding of risk for obesity and disease.

How will you look to maximise the outputs of this work?

We will maximise the output of our work by collaborating extensively with research groups bringing experience of human clinical genetics. This interaction will enhance our ability to identify genetic mutations that are associated with obesity. It will be this clinically relevant information which we use to develop models in zebrafish. We will make our findings publicly available through open access publications, and will present our work at local, national and international scientific meetings. We will work closely with funders (including the MRC and also medical charities) to disseminate our research findings to the general public.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 35,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using zebrafish as a model system to understand how genetics and environment contribute to obesity and obesity-related disease. Zebrafish are an excellent model for studying obesity and related disease and have extensive genetic and physiological similarities to humans. These similarities mean that disease causing mechanisms are often shared between zebrafish and humans and so the information we uncover will be

relevant for human disease processes. This high level of conservation also means that any drugs identified to treat disease processes in zebrafish might also be applicable to humans. In addition to similar disease processes, zebrafish are also a good model system because they offer a robust and tractable model to test the function of obesity genes – it is relatively straightforward to genetically alter zebrafish and test very specific obesity gene mutations. Further, zebrafish are highly amenable to imaging tissues that are implicated in obesity. For example, we can see into body fat to understand how it responds to obesity, and we can see into the brain to understand how they might influence appetite and obesity susceptibility. We mainly use young zebrafish at the larval and juvenile post-embryonic stages which provide a simple model and allow us to test our specific ideas in a relatively simple system. Together, these advantages of the zebrafish model allow us to obtain useful information on obesity genes and susceptibility to obesity-associated diseases.

Typically, what will be done to an animal used in your project?

One of the main advantages of using the zebrafish model is that we can conduct non-invasive imaging of tissues implicated in obesity and obesity-related disease. With this in mind, the majority of animals in this project will be genetically altered to encode fluorescent proteins in their genomes which allow us to visualise specific cells and tissues that are relevant to obesity and associated disease (e.g., fat cells or neurons that control appetite). Zebrafish will also be genetically altered to carry potential obesity- causing mutations that have been identified from human clinical and genetic studies. By using these two types of genetic alteration we will be able to test whether a specific mutation/genotype affects obesity or disease and visualise the process in cells and tissues. This methodology will allow us to gain new information on these obesity genes. In addition, we will also perform diet manipulations to test whether for example exposure to a high-fat diet or over-feeding will modify the function of the obesity genes.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of our experiments will be non-invasive and without any signs of adverse effects for the zebrafish. With a small proportion of the diet manipulations, we will be inducing a mild form of pre- diabetes characterised by increased blood sugar levels. It is important that we induce these very early signs of diabetes and obesity-related disease as we wish to understand the physiological response to this diet. However, these are very early signs of disease without any strong adverse effects and experiments will be halted prior to any strong adverse effects within a few weeks of the onset of symptoms. For some of the diet manipulations we may induce weight loss by food restriction. However, zebrafish are remarkably resilient to food restriction and no adverse effects are observed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We do not study animals with severe adverse effects. However, prolonged administration of a high-fat diet can induce increased blood glucose and insulin resistance which we would classify as moderate adverse effects. Even though we are inducing these symptoms, we would study these animals over a relatively short period of time so that any damage to organs or tissues would not manifest and disease severity would not progress. We expect that less than 1% of animals which are part of this project would experience moderate adverse effects. We study animals with mild adverse effects, such as mild stress or physical restraint to facilitate imaging. However, a relatively low percentage of animals would experience mild adverse effects (less than 5%). The vast majority of animals will not experience any detectable adverse effects (greater than 90%) and most are kept for breeding and maintenance with no evidence for suffering or adverse effects.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Obesity is a complex physiological trait involving cross-talk between multiple organ systems. For example, mutations that influence the function of neurons within the brain may control our appetite and how much we eat. Whereas, mutations which function within fat cells may govern how the fat cells respond to obesity. The cross-talk between these systems determines our susceptibility and response to obesity. As such, it is difficult to effectively model these responses in vitro in a culture dish. However, in zebrafish we use a relatively simple model to understand these interactions but which faithfully recapitulate interactions that occur in obesity.

Which non-animal alternatives did you consider for use in this project?

In this project, we will also utilize culture of human and mammalian cell lines wherever possible. Cell culture systems are very useful for studying responses in single cell types and autonomous responses. However, to properly understand obesity and body fat levels, composition and distribution, laboratory animals must be used that recapitulate the interactions that occur in the physiological context of a highly complex living system.

Why were they not suitable?

To properly understand how genotype and diet interact to regulate body fat levels and distribution it is necessary to conduct in vivo experiments and model systems that recapitulate the interactions that occur in the physiological context of a highly complex living system. For example, for studying fat distribution it is not possible to culture multiple fat types together and assess interactions leading to fat distribution differences in obesity – this must be done in a living physiological system.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have calculated the number of animals that we are likely to use based on our current use of zebrafish as a model system. Of the animals we project to use, the majority are for breeding and maintenance purposes (PROTOCOL 1). We have calculated the projected number of fish, based on our current use of approximately 150 tanks of zebrafish per week, in which we keep an average of 20 fish. We aim to refresh stocks once per year, meaning that we will use 5 separate generations of each stock over the course of the project, giving a total of 15,000 animals.

For PROTOCOL 2 we project use of up to 6,000 zebrafish for the generation of new genetically altered animals. This number largely reflects the new ability to target gene function at scale using new tools, including CRISPR/Cas9 gene editing. We plan to test the effects of 250 genes over the course of this project using an average of 20 animals per assay – this will require 5,000 animals. We will also generate stable mutant lines from genes that exhibit particularly important functions when assessed by acute gene editing, and expect to generate up to 20 such lines, with current estimations that we need to grow up 50 animals to successfully do so (1000 animals). Thus, we estimate that we may use up to 6,000 animals in total for PROTOCOL 2 .

In addition, we predict using a further 8,000 animals for our experimental analyses (PROTOCOL 3). We plan to use 8,000 animals for live imaging, photostimulation and induction studies as part of PROTOCOL 3 and this estimate is calculated based on current usage of analysing ~80 experimental groups of ~20 animals over the course of this 5-year project.

For PROTOCOL 4 (diet manipulations), we plan to use 6,000 animals based on current usage. Of these 6,000 animals, we estimate that 3,000 will be used for immersion in a high-fat diet solution (25 experiments using 25 animals per group), and 1,000 animals used each for the food restriction, overfeeding and high-fat powder diet manipulations (8

experiments using 25 animals per experimental group). We carry out very careful calculations to define how many animals are needed to find statistically meaningful effects in our experiments, and will continue to do so for new studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Firstly, wherever possible we will utilize zebrafish at unprotected stages (5 days old and younger) to reduce the number of animals on experimental protocols. For example, we can visualise the neurons in the brain that influence appetite at 4-5 days old and we have shown that manipulation at these stages can influence feeding almost immediately. Secondly, we will control experimental variability by utilising precisely stage-matched individuals for experimental comparisons. We have established criteria for these stages previously and these are now standard for the field. We will also control fixed effects such as housing, diets and husbandry by consulting with professional husbandry staff and using well characterised inbred zebrafish strains. Thirdly, in the diet manipulation we make use of randomisation to distribute inescapable differences between individuals randomly across the groups. Fourth, we will make use of non-invasive longitudinal imaging of zebrafish which allows us to perform a repeated measures experimental design. By taking two or more measurements for each animal over time we can effectively control for inter-individual variation, improve experimental efficiency and reduce animal numbers.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will work closely with other research groups to ensure the efficiency of our transgenesis and mutagenesis is optimal. By doing so, we will keep up-to-speed with the latest technological developments and improvements in efficiency which will ultimately reduce animal usage. We will also work closely with professional husbandry staff who are optimising diet and other husbandry procedures to minimise variability across fish stocks and ultimately reduce the need to raise large numbers of fish for experiments. Where possible we will conduct pilot experiments to ascertain exact experimental conditions and sizes of effects which will enable us to more accurately predict sample sizes needed and will ultimately reduce use of zebrafish.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are using the zebrafish model to understand how genetics and environment interact to regulate obesity and associated disease. Zebrafish are an ideal model system to study obesity and related disease owing to a high degree of conservation to mammalian disease processes, coupled the ability to non-invasively image relevant fat cell types in living animals without perturbing the endogenous physiological context. This experimental advantage represents a significant refinement of methodology as equivalent techniques in mammalian models requires surgical implantation of an imaging window which not only causes suffering and distress to the animal but also initiates an inflammatory response. Owing to zebrafish transparency and their small size, the imaging method we employ is entirely non- invasive. An additional refinement is the simplicity and high efficiency of genetic manipulation in zebrafish. These methodologies allow us to generate transgenic lines that enable to visualisation of specific cell and tissue types, and also allow us to engineer specific mutations into the zebrafish genome that allow us to model human obesity mutations. These methods are non-invasive and can be conducted in the externally fertilised eggs, with any potential adverse effects owing to the methodology ascertained prior to reaching protected stages. To aid further refinement we will continue to research new methodologies and work together with husbandry staff to develop and implement new practices that bring refinement to existing techniques.

Why can’t you use animals that are less sentient?

Body fat and regional adiposity is a bony fish innovation (ie, it evolved along with a bony skeleton). Therefore, zebrafish are one of the simplest and less sentient organisms which possess regional body fat. Further, we use zebrafish at the earliest stages where body fat appears and so are arguably less sentient.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The vast majority of our experiments are non-invasive and do not cause any adverse effects. However, we implement a robust monitoring system to record and monitor any potential adverse reactions. For example, when conducting diet manipulations we keep a daily monitoring record along with multiple checks throughout the day on the welfare of the animals on the special diet. This allows us to respond quickly and effectively to any adverse effects. We will continue to revise and improve on the monitoring.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow and implement the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines issued by the NC3Rs. Further, we will keep abreast of developments

in the scientific literature which will be the best source to identify refinements to existing methods.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We follow news from the National Centre for the Replacement Refinement & Reduction of Animals in Research (NC3Rs), and also discuss with local vets and named animal care and welfare officers.

Importantly, we stay informed of the relevant scientific literature for methodology development and refinements.

34. IBV: Attenuation and Vaccine Development

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

chicken, IBV, Coronavirus, pathogenicity, immunogenicity

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	adult, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Infectious bronchitis virus (IBV) causes a respiratory disease in chickens. It causes economic losses and is a risk to food security as infected chickens do not gain as much weight or produce as many good quality eggs as uninfected chickens. IBV is prevalent throughout the world and there are many different strains of the virus circulating. Currently available live attenuated vaccines are not able to protect chickens against all of these strains and have the potential to revert to a more virulent form.

The aim of this project is to produce a rationally attenuated IBV vaccine vector that can be modified for the production of effective vaccines against a variety of IBV strains belonging to different serotypes. In order to achieve this aim, we have three objectives:

1. To identify pathogenic determinants present in the IBV genome.

2. To identify immunogenic determinants present in the IBV genome.

3. To determine the protective efficacy of IBV vaccine candidates.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The avian coronavirus, infectious bronchitis virus (IBV), is a highly contagious poultry pathogen prevalent in all types of poultry flocks worldwide and is responsible for economic losses, welfare problems in chickens and a potential risk to food security. IBV preferentially causes respiratory disease, but can also infect other organs such as the kidneys (resulting in kidney disease) or the reproductive tract (resulting in loss of egg production and/or egg quality).

IBV is the aietological agent of infectious bronchitis; an economically important infectious disease affecting chickens in the UK. It is estimated that IBV affects 22 million chickens and costs the UK poultry industry £23 million every year. Poultry is an important food source worldwide; with an estimated 55 billion chickens produced worldwide per annum, including 5 billion for egg production.

What outputs do you think you will see at the end of this project?

Outputs of this work will include new information regarding pathogenic and immunogenic determinants within the IBV genome. Outputs may include the generation of genetically modified IBVs that have potential to act as live attenuated vaccines against multiple serotypes. We always aims to publish our work in scientific journals in a time appropriate manner therefore outputs will also include publications. This ensures the scientific community remains up to date with the results that we produce. We will also file patents for any results that may have commercial potential/impact.

Who or what will benefit from these outputs, and how?

Our work uses the host species that IBV affects and therefore is of direct benefit for the development of IBV vaccines to protect chickens against this disease. All commercial chickens are vaccinated against IBV. Vaccine breakdown has a major effect on the UK poultry industry, not only financially, costing £23 million every year, but also affecting bird

welfare and risking food security. This work will establish whether our novel approach to vaccine design through rational attenuation and modification of vaccine serotype is capable of producing safe and efficacious vaccines for the control of IBV that are less likely to revert to virulence. The development of safer vaccines will reduce the amount of antibiotics used to treat secondary bacterial infections associated with infectious bronchitis, which would have positive environmental impact. Results of these studies may reveal correlates of protection against IBV, informing the design of future studies.

In a shorter time scale, this project will allow for the identification of pathogenic and immunogenic determinants within the IBV genome. This is not only specific for the IBV field of research but will also prove information for the wider coronavirus field, and therefore may aid in vaccine research and design for other coronaviruses. The coronavirus family consists of a large number of viruses that infect a diverse range of species including swine, bovine and humans. The outputs of this project will therefore feed into the One Health initiative. The results of this project will be of direct benefit to the poultry industry and vaccine developers.

How will you look to maximise the outputs of this work?

This study will inform the wider academic community including other researchers working in the fields of molecular virology, livestock health and coronavirus research, particularly IBV, and will inform approaches to development of other veterinary and human vaccines. Knowledge generated by this project will be widely disseminated to the research community through peer-reviewed publications and presentations at national and international virology conferences and interactions with members of the poultry industry and veterinarians. We will aim to set up collaborations with both academic partners and industry partner when appropriate. We will openly discuss and aim to publish “negative” data which in the regard of this project involves the identification of genome factors which are not pathogenic or immunogenic determinants.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 1750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using the natural host of IBV, the domestic chicken for which we wish to contribute to better IB disease control. Only the chicken can be used for propagating most strains of IBV, assessment of pathogenicity and analysing potential vaccines; we are developing vaccines for protecting chickens against IBV.

Typically, what will be done to an animal used in your project?

This project will include two main types of experiments. The first, to determine pathogenicity will involve birds being inoculated/vaccinated via the intraocular (eye drop) and/or intranasal route with IBV. Chickens will be monitored twice daily. Clinical signs including snicking (sneeze) and rales (tracheal vibrations) will be assessed in a non invasive way for up to 10 days post infection. Swabs of the oral cavity, oesophagus and trachea maybe taken daily. Typically, at either 4 or 6 days post inoculation randomly selected birds will be euthanised. The second type of experiment to assess immunogenicity or vaccine efficacy will begin as described for the first experiment. This experiment will differ as chickens will typically be challenged three weeks post vaccination. The challenge virus will be a pathogenic IBV and will also be administered via the intraocular (eye drop) and/or intranasal route.

Chickens will be monitored both post vaccination and post challenge for up to 10 days. Blood samples will typically be taken from a superficial vein (e.g. wing vein) two days pre vaccination and two days pre challenge. Typically, at either 4 or 6 days post challenge randomly selected birds will be euthanised. All birds will be culled at the end of both experiments as described; the end of the experiment will be 14 days post the last inoculation.

What are the expected impacts and/or adverse effects for the animals during your project?

All our experiments using chickens involve the development of vaccines ultimately for the benefit and protection of chickens against disease and are carried out in environmentally controlled experimental animal facilities. We keep animals under regular observation and use non-invasive measurement of clinical signs of infection. IBV mainly causes clinical signs very similar to the common cold in humans; a few days of snicking (akin to sneezing), watery eyes and wheezing. Chickens are expected to recover from respiratory disease within ten days and will experience a maximum of moderate disease severity, most will only experience mild disease. As we need to analyse virus growth and disease pathology in different organs, all chickens will be humanely euthanised at the end of the experiment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Chickens are expected to recover from respiratory disease within ten days and will experience a maximum of moderate disease severity, most will only experience mild disease. It is estimated that 60% of chickens will experience mild severity and 40% moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Pathogenicity and vaccine efficacy can only be assessed using the natural host of IBV, chickens; there is no in vitro or ex vivo alternative model. Embryos cannot be used as alternatives to chickens as even strains non-pathogenic for hatched birds can cause morbidity in embryos. Also, although the non- pathogenic Beaudette strain does cause ciliostasis when inoculated onto ex vivo tracheal organ cultures (TOCs) in the laboratory, it does not cause tracheal ciliostasis following eyedrop/nasal inoculation of chickens. In vitro assessment of immunogenicity, for example virus neutralization assays will be carried out where ever possible, however the identification of virus neutralizing antibodies in vitro has been shown not to correlate with protection in vivo.

Which non-animal alternatives did you consider for use in this project?

Tissue culture, including primary chicken kidney cells and ex vivo tracheal organ cultures (TOCs) will be used whenever possible. The growth and titration of IBV isolates that cannot be grown in cell culture or TOCs will be carried out using embryonated eggs.

Why were they not suitable?

Pathogenicity, immunogenicity and vaccine efficacy can only be assessed using the natural host of IBV, chickens; there is no in vitro or ex vivo alternative model. As stated above, although the non- pathogenic Beaudette strain does cause ciliostasis when inoculated onto ex vivo TOCs in the laboratory suggesting that it may be pathogenic, it does not cause tracheal ciliostasis following intraocular (eye drop) and intranasal inoculation of chickens. Additionally the H120 and Beaudette strains of IBV which are both attenuated in vivo, are pathogenic in embryos.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

A statistician is consulted prior to each study to ensure that an appropriate number of chickens is used to generate meaningful results. The number of birds per group at each time point is selected to guarantee statistically relevant results for the assessment of protection and pathogenicity based on many years of experimental work on IBV and the recently published meta-analysis of the required sample size in vaccination-challenge experiments with IBV.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have consulted the publication on the meta-analysis of the required sample size in vaccination- challenge experiments with IBV and we have taken into account the results from the many years of IBV in vivo studies that have been carried out.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continually review the published literature with a statistician to ensure optimal number of chickens are being used. We will also keep up to date with the standards set for IBV vaccination by the European Pharmacoepia. Post-mortem tissues will be shared with other researchers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are using the natural host of IBV, the domestic chicken for which we wish to contribute to better IB disease control. Only the chicken can be used for the assessment of pathogenicity and analysing potential vaccines; we are developing vaccines for protecting chickens against IBV. Most of the chicks that we infect develop mild respiratory disease from which they recover within a few days. Chickens will be observed twice daily following inoculation with IBV to ensure that clinical signs and welfare of the chickens are closely monitored. Inoculation of the chickens via the intraocular (eye drop) and intranasal route mimics both spray vaccination practices and natural infection via the aerosol route.

Chickens inoculated in this manner only experience mild momentarily discomfort.

Why can’t you use animals that are less sentient?

Embryonated eggs will be used for the growth of IBV isolates that cannot be grown in cell culture. Cell culture and TOCs will be used where possible to minimise the use of embryonated eggs.

Pathogenicity and vaccine efficacy can only be assessed using the natural host of IBV, chickens; there is no in vitro or ex vivo alternative model. Embryos cannot be used as alternatives to chickens as even strains non-pathogenic for hatched birds including Beaudette and H120, can cause morbidity in embryos. Tracheal ciliary activity in ex vivo tracheal organ cultures is also not an appropriate model for pathogenesis in vivo as IBV strains such as Beaudette can cause ciliostasis when inoculated onto ex vivo TOCs in the laboratory but cannot not cause tracheal ciliostasis following intraocular (eyedrop) and intranasal inoculation of chickens.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our procedure for determining pathogenicity and vaccine efficacy has been used for many years by us and others, and has been published. We inoculate the chickens via the intraocular (eye drop) and intranasal route, mimicking spray vaccination and aerosol transmission but in a more controlled way.

Chickens will be monitored twice daily following inoculation with IBV to ensure that clinical signs and welfare of the chickens are closely observed. The chickens used in this research will be housed in open raised floor pens with solid floors which were specifically designed in consultation with our Animal Technicians and NACWOs. Flexibility was an important feature of the design to ensure pen sizes can be increased as the birds grow. Chickens will be provided with enrichment including perches, pecking blocks and live feed. Foraging, scratching and pecking are all important behaviours to birds and so enrichment provided will enable them to express their species specific behaviour.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will adhere to the ARRIVE guidelines for reporting of in vivo studies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep updated with published literature regarding animal experimentation in poultry and will regularly consult the NC3Rs website. We will maintain an open dialogue with the animal technicians and NACWOs in relation to enrichment that can be provided.

35. Developing New Multivalent Recombinant Vaccines Platforms Against Infectious Diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Vaccine, Infection, Prevention, Immunology

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of the research is to develop new and improved vaccines. These will be low cost vaccines that can protect against several disease that affect children and adults in Low-Middle-Income countries (LMIC) and in the Western world.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There is an urgent need for improved vaccines against infectious diseases. New vaccines need to be safe, well tolerated, effective and offer broad coverage against many pathogens. In fact, multiple pathogens often coexist in the same geographical areas and a single vaccine able to control these diseases would be a desirable asset.

There is also an urgent need to make vaccines that are easy to develop and produce at low cost, to benefit poorer countries via increased affordability.

It is important to develop vaccines that are based on simple construction and therefore suitable to move from early experimentation to production in the shortest possible time. The design and realisation of new vaccines should be simple enough to allow modifications that increase protection against multiple pathogens in response to epidemiological changes and also to allow preparedness for the potential emergence of new and unknown diseases with pandemic potential.

What outputs do you think you will see at the end of this project?

The main output of the project will be the generation of new, safe, effective, versatile, vaccines against bacteria that cause grave infections worldwide in adults and children. The vaccines will protect simultaneously against many variants of each bacterial pathogen, given that these variants often coexist in the same geographical area or circulate between different areas, emerging suddenly and unpredictably. The project will yield vaccines mainly, but not exclusively, against bacteria that cause acute infections for which antibiotic treatment often fails. The project will target mainly bacterial meningitis, gonorrhoea, salmonellosis and can be extended to other infections caused by bacteria, virus and parasites.

The information produced by the project will be divulged in due course for the benefit of the scientific community. This will happen in the form of scientific publications and presentations at meetings.

The work will produce intellectual property that will support in the future grant and patent application and clinical trials.

We will also engage with the general public, via the University press office, the engagement channels of our industrial collaborators, via science festivals and informal public engagement talks and via appropriate contacts with the press and the media.

Who or what will benefit from these outputs, and how?

The project will primarily benefit adults and children at risk of acute and chronic infections caused by meningococcus, gonococcus and Salmonella. These diseases are widespread globally with an unpredictable epidemiology and devastating epidemic potential especially in Low and Middle Income countries of Africa and East Asia. These diseases have a very high level of resistance to antibiotics and therefore their prevention through large-scale vaccination programmes remains the best option at a global level.

The short-term output (3-5 years from commencement of the work) will be the construction, development and testing at the preclinical level (to the end of animal experimentation and ready to enter studies in humans) of candidate vaccines able to protect against multiple variants of each one of these pathogens.

This will be followed by the necessary steps for the production of clinical batches of the vaccines, licensure and organisation of clinical trials in humans (5-6 years from commencement of the work). Trials in humans will follow and will be completed within 6-10 years from commencement of the work.

Given the recent global experience with vaccines against COVID-19, and the current collaborations that we have with industrial partners, these timelines may be shortened to deliver the vaccines.

How will you look to maximise the outputs of this work?

We collaborate with other academic institutions and with industrial partners. This will allow us to expedite the research phases of our work and also to perform the essential steps required by the future development, testing and production of the vaccines. Therefore, the outputs of this research will be impactful. We will divulge our work via open access publications and talks at conferences subject to prior protection of intellectual property if required. We will engage with the public to share and explain our research via open days, interaction with the media and suitable press releases from the University.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We shall use adult mice to test the immunogenicity of the vaccines. The mouse model is a reliable and broadly used small animal model for the initial evaluation of immune responses and protection induced by vaccines against many bacterial pathogens. Results

obtained in mice have informed vaccine development against many pathogens of humans and other domestic animals for decades.

Typically, what will be done to an animal used in your project?

Vaccines will be injected into animals. They may be injected intravenously (i.e. into a vein), intraperitoneally (i.e. into a body cavity), subcutaneously (i.e. under the skin), intranasally (i.e. instillation of small volumes into the nostrils), orally (i.e. into the mouth or stomach). Booster doses may be given at 2-4 weeks intervals, usually for a maximum of 4 doses, to mimic the number of doses that will be given to humans if the vaccines were successful in the later clinical phases.

Blood samples will be taken after each vaccination to test for the induction of immune responses.

In some cases, animals will also be injected with drugs that act on the bacteria (e.g. antibiotics) or that increase immunity (e.g. antibodies).

In some cases, vaccinated animals and a small number of unvaccinated control animals will be infected for a short time with low, subclinical, doses of live bacteria.

At the end of the experiments the animals will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

The vaccines will consist mainly of inactive preparations and therefore cannot cause infections. In some cases we may use live bacteria that are genetically impaired in their ability to grow in a mammalian host. The vaccines will have been constructed and previously tested in non-animal models (e.g. cell culture, chemical tests etc.) to make sure that they are likely to have the lowest possible level of side effects.

Our experience on immunity to bacteria and vaccine development, acquired during several decades of fundamental research, allows us to predict correlations between the type of immune responses induced by vaccines and the presence or absence of protection. In the case of Neisseria vaccines, serological tests (i.e. serum bactericidal activity, SBA) can be taken as predictors of vaccine efficacy.

Therefore, only in rare cases we will need to re-infect vaccinated animals with virulent pathogens to conclusively determine vaccine efficacy. This will happen, within this project, in the case of Salmonella infections where a firm and conclusive immunological correlate of protection is not available. When this is done, the infection experiments with live bacteria will be performed at low doses and their duration will be short, thus minimising clinical signs, should the vaccine prove not to be sufficiently efficacious.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice:

- Mild=96%
- Moderate=4%
- Severe= 0%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals are needed to test the quantity and types of immune responses induced by the vaccines that we are currently constructing. In some cases, we also need to infect animals with low doses of live bacteria to ensure that vaccines can protect animals against disease. This is the necessary prelude to human testing in clinical trials.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives to conclusively test the immunogenicity and protection of vaccines. These need to be ultimately tested within the complexity of the immune system of a whole mammalian organism.

We routinely use non-animal alternatives to test the side effects of our vaccine preparations, to determine their chemical composition, measure the presence and quantity protective components within the vaccine. This is done before any animal experimentation takes place.

We use cells in culture, chemical tests that measure the composition of the vaccine and immunological tests that predict the efficacy immune responses to the vaccine. For example, in the case of Neisseria vaccines, we measure the ability of animal sera to kill bacteria as an indirect measure of vaccine activity and protection.

Why were they not suitable?

Because it is impossible to evaluate if a vaccine stimulates immune responses unless a whole animal is injected with a vaccine. Non-animal tests (see above) can in some cases be used to measure the presence of immune responses induced by the vaccine in the animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have 30 years of experience in designing animal experiments with the aim of testing vaccines against bacterial infections.

We estimated the numbers of animals according to the expected number of vaccines that we shall test, considering groups sizes and experimental protocols. The numbers of animals within each experimental group and the size of each experimental group are decided as indicated in the sections below. Briefly, throughout the project we estimate to test approximately 200 possible candidate vaccines in small-scale experiments involving 10 mice per candidate on average. We expect to take forwards about 10% of these (20 candidates) for further immunogenicity and functional studies that will involve approximately 150 mice per candidate vaccine.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We design experiments to include as many experimental groups in parallel as possible, with the smallest number of animals per group (as determined by statistics, pilot experiments and mathematical modelling); we associate one control group to as many experimental groups as possible so to reduce the number of control animals used in our experiments. We plan to make the best possible use of experimental design assistants including the NC3R one (Experimental Design Assistant, NC3R) whenever appropriate.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We carry out pilot studies using small numbers of animals, but making sure that these group sizes can support power calculations for larger scale experiments and/or yield statistically significant results able to provide a definitive answer on whether to take forward or dismiss a particular vaccine candidate. In our work we plan to take forwards to larger animal studies only those vaccines that in pilot experiments show real promise.

Based on pilot studies and on similar work performed by us in the past, we perform statistical calculations to determine the minimal group sizes and number of repeats without compromising scientific accuracy.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We shall use the mouse model of vaccination and reinfection. Animals will be immunised either with non-living bacterial vaccines or with live attenuated bacteria. The reactogenicity (side effects) of the vaccines will be extensively reduced by genetic manipulation of their DNA and is ascertained by extensive biochemical characterization and in vitro testing on cell lines. We have adopted the use of commercially available reporter cell lines to ascertain and measure the reduction in toxicity of the vaccines prior to inoculation into animals. We use cells lines created specifically to investigate TLR4 activation. Whenever possible, we use antibody levels and the presence of cellular responses as correlates of vaccine-induced protection (e.g. in the case of meningococcal and gonococcal vaccines); this abrogates the need for re-challenge with virulent bacteria. In the case of other bacterial pathogens, such as Salmonella, it is necessary to infect the animals with live bacteria to conclusively ascertain vaccine efficacy. In this case we only perform reinfections in those animals immunised with vaccines that have proven immunogenic in in vitro tests (i.e. measurements of antibodies and/or cellular immunity). When reinfection is required, we use low doses of live bacteria and we allow the infection to progress only for the time necessary to detect differences in bacterial numbers between vaccinated animals and non-vaccinated controls. We precisely know the grow rates of our challenge Salmonella strains and therefore we can be sure that the infection would not proceed faster than expected even in the event the vaccines proved to be ineffective and/or in non-immunised controls. Therefore, the challenge infections largely remain within the mild band of severity.

Why can’t you use animals that are less sentient?

Vaccine testing requires adult animals with a mature immune system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We provide the best possible husbandry care for our animals. This includes regular cleaning, health checks, within-cage enrichment tools and the use of female animals as much as possible to minimise the chances of in-cage fighting and therefore allowing group housing. We acclimatise our animals for at least 7 days before the start of any experiments. We regularly inspect the animals before and during the experimentation to ascertain that they stay in within the severity of the ascribed protocol.

Experimental procedures are performed by skilled PIL (Personal Licence) holders, according to tried and tested protocols that have evolved during several decades to reduce stress and suffering to the animals. Refinements include optimisation of volumes, use individual needles for each animal, optimisation of needle size, improvement of techniques for gavage through the use of light anesthesia

that allows more accurate dosing and reproducibility of results. We reduce the potential toxicity of our candidate vaccines by removing from the bacterial DNA specific genes (e.g. lpxL1, msbB) that encode for parts of their membrane that have inflammatory activity. When we infect animals with live bacteria we always use low numbers that do not cause disease and we do not allow the experimental infections to progress to numbers of bacteria in the body that can cause, at worst, mild clinical symptoms. The numbers of bacteria to be injected are precisely measured in the inoculum by optical density and then confirmed by counting viable bacteria on bacteriological growth media. Gavage procedures performed under light anaesthesia (with rapid recovery) ensure reproducibility of experiments, reduced variability (thus need to use less animals per group) and reduced stress/harm to the animal due minimal chances of surface damage of the aesophagous (potential consequence of an animal resisting or struggling during the gavage procedure). Welfare assessment protocols will be considered when appropriate, as these can be useful tools to monitor adverse effects and determine when humane endpoints have been reached. The experience of the Named Persons and animal technicians will be useful to create welfare scores by recording relevant adverse effects and incorporating the earliest humane endpoints.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the PREPARE (https://norecopa.no/prepare) and ARRIVE (https://arriveguidelines.org) guidelines as a routine of good practice and as a requirement for scientific publications. In addition to these, when appropriate and required we shall also benefit from papers from the Laboratory Animal Science Association, (LASA) https://www.lasa.co.uk/current_publications/. The details of each experiment (e.g. dosing regimens) will be based, whenever possible, on peer reviewed publications that have used similar vaccines in the past. Many of these papers are from members of our research group.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our establishment provides regular updates on the 3Rs. The PPL holder is the Chairman of the Animal User's committee at his establishment and a member of the establishment Management Committee.

These committee meetings include a dedicated section where improvements of the 3Rs are discussed. The PPL holder and his group attend NC3R events and workshops and subscribe to the NC3Rs e- newsletter. Regular consideration and reflection of the latest practical guidance from Laboratory Animal Science Association (LASA) will provide additional sources of new recommendations and advances in animal techniques.

36. Improved Induction and Identification of Tinnitus in Mice Prior to Treatment with Drugs or Genetic Alteration

Project duration

5 years 0 months

Project purpose

Basic research

Translational or applied research with one of the following aims:

- Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

tinnitus, salicylate injections, noise exposure, genetic manipulation, behavioural test

Animal types	Life stages
Mice	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim is to use refined models of tinnitus in mice to identify receptors and test drugs that might produce a significant reduction in the tinnitus percept.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is estimated that over four million prescriptions a year (in the USA and Europe) are for drugs to treat symptoms associated with tinnitus such as stress or problems sleeping. There is currently no drug available to abolish the perception of tinnitus directly and this represents a potentially huge market.

The current healthcare bill for treating tinnitus on the NHS is estimated to be 750 million pounds per year. There have been many attempts to find a pharmacologically effective treatment for tinnitus, in the last 10 years, but so far no drug has been approved for its treatment. Tinnitus is a symptom in a wide variety of clinical histories and there is clear evidence that more than one mechanism underlies tinnitus. Fundamentally we do not fully understand tinnitus and have no broadly effective way to treat it. Tinnitus has similarities to the chronic pain associated with phantom sensations experienced after limb removal and a new drug class has recently been shown to be effective against this type of neuropathic pain. We are hoping to develop reliable models of tinnitus in mice that can then be used to show that this same drug class is effective against various subtypes of tinnitus, especially those that are linked to hearing impairment. Mice are important in this research because genetic knockouts have been produced that will allow investigation of the mechanisms underlying tinnitus.

What outputs do you think you will see at the end of this project?

The drugs we are proposing to use were designed for treating heart problems or neuropathic pain and we hope to obtain reliable evidence that this type of drug is also effective in alleviating the symptoms of tinnitus, once we have refined our mouse models of tinnitus. On the basis of our initial methodological work we expect to be able to produce one peer reviewed paper on the use of characteristic body movements associated with the acoustic startle reflex as the basis of a reliable method for detecting tinnitus in mice. We will then test the most promising examples of the drugs in our mouse models. As some of the drugs are nearing the end of their patent protection, then we would not expect any delay being placed on the publication of a second paper aiming to show that this type of drug is effective in reducing the symptoms of tinnitus.

Who or what will benefit from these outputs, and how?

There is a clear need for a pharmacological treatment for tinnitus and if we can establish a reliable model for testing drugs in mice then this should be generally useful for the initial testing of drugs with therapeutic potential. Our commercial partner wants us to test drugs that they have developed for treating conditions such as neuropathic pain. They are now interested in repurposing the drugs as a potential treatment for tinnitus. Before initiating expensive clinical trials they need convincing evidence of efficacy in animal models. Other

drug development programmes would also benefit from having a reliable mouse model for tinnitus.

How will you look to maximise the outputs of this work?

This work involves collaboration with a large drug company. Once we have established an improved tinnitus model in mice we would encourage the company to repurpose these existing drugs as a treatment for tinnitus. They have the resources to initiate clinical trials and this would be the next step.

Species and numbers of animals expected to be used

- Mice: 300 wildtype and 50 genetically altered (GA)

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Tinnitus does occur in children, but it is more common in adults and its incidence peaks in the population group aged from 60 - 70 years old. There is no known direct genetic cause and in many people the exact causative factors are unknown. Thus the most appropriate animal model for drug testing is a mature mammal. The strain of mice to be used in this study (C57BL/6J) shows early onset hearing loss which is already present by six months and is equivalent to the age-related hearing loss (presbycusis) found in humans of over 60 years old. Thus, in order to have time to induce tinnitus and treat it with various drugs, before the onset of hearing loss, we will start the baseline behavioural training while they are still juveniles. Mice have a brain that has the same structures and neurochemicals as all other mammals and are a suitable model of the human auditory system. The main advantage of mice is the availability of technology designed to abolish the expression of specific genes in particular parts of the body, such as the ear. This allows us to test the role of particular gene products (such as ion channels involved in the activity of the auditory nerve). We, along with other scientists, aim to develop a behavioural test for tinnitus in mice that is relatively quick and easy to implement. This behavioural test is designed to allow us to test the effects of deleting certain gene products that may be essential to the development of tinnitus. We will also test drugs that may alleviate the symptoms of tinnitus by blocking the gene product without any genetic manipulation.

Typically, what will be done to an animal used in your project?

We have two models of tinnitus:

1) The salicylate model is a rapid onset, reversible form of acute tinnitus that involves the injection of a high dose of sodium salicylate (the active form of aspirin). This form of

tinnitus occurs in human patients who receive high doses of oral aspirin to treat certain types of advanced cancer. At high doses salicylate (aspirin) is an effective pain-killer and anti-inflammatory agent. The main side effects of these high doses of salicylate are mild hearing loss and tinnitus along with thinning of the blood (anti- coagulant function) and possibly irritation of the stomach/intestine. Salicylate reliably produces tinnitus in 95% of animals and its effect peaks after about two hours. We can then compare the evidence for tinnitus in the wildtype mice with those that have had the gene for a specific membrane channel (HCN2) deleted. This testing involves presenting a series of brief startling clicks to the animal and measuring small body movements using a system of infrared cameras that track the relative ear and trunk, neck or tail movements in 3D space as the animal moves freely around the chamber. After 60 min. of testing we have sufficient data to determine if the animal has developed evidence of tinnitus and the experiment is concluded. We would not expect any of the GA mice to show evidence of tinnitus. We have not used salicylate to induce tinnitus in mice before but it is a standard model in mice. The first experiments will gather pilot data to check the safety of our protocol for administering the salicylate and the ivabradine and experimental drugs used to treat the tinnitus symptoms. Only one injection of salicylate will be made per mouse and the experiment will end within 5 hours.

2) The noise exposure model is a chronic model of tinnitus that takes about 6 weeks to develop in mice and involves two, 1 hour long periods of moderate noise exposure applied two weeks apart. The sound is presented to one ear using a loudspeaker connected by a tube to the animal's left ear while it is deeply anaesthetised. During the period of anaesthesia, we record auditory brainstem responses (ABRs) using subcutaneous needles. These needles pick up tiny brain potentials that we can use to measure hearing thresholds, before and after the noise exposure, to confirm the degree of hearing loss. Anaesthesia is usually induced by an intraperitoneal injection of a liquid anaesthetic mixture followed by supplementary injections to maintain anaesthesia at a constant deep level. Gaseous anaesthetic gives greater control over anaesthetic depth and would be used if it were possible to supply it safely without interfering with the experiment. Currently we are unable to safely extract it from the sealed acoustic chamber without introducing noise into the chamber during the ABRs, but if it becomes possible to do so in future then we would use an anaesthetic such as isoflurane under the direction of the NVS. Animals are anaesthetised for about two hours and then recover over the following two hours after one or two sessions of noise exposure. The animals may demonstrate hearing loss in one ear and are left for the next six weeks before behavioural testing is started. About 40-50% of the noise exposed animals should develop tinnitus in the wildtype population but we would expect that none of the GA mice would develop tinnitus if our hypothesis is correct. As it is chronic tinnitus a range of drug doses will be used to test drugs that block the channels that have been deleted in the GA mice. All the tinnitus animals and some of the GA mice would also be given sham drug injections (no drug just the vehicle that they are dissolved in) to act as controls and check for tolerability issues. These animals would then be tested with the GPIAS (gap-induced pre-pulse inhibition of the acoustic startle)

behavioural test to confirm whether or not the sham drug injection (vehicle only) had any effect on behaviour. The experiment would then be concluded.

What are the expected impacts and/or adverse effects for the animals during your project?

High concentrations of salicylate cause tissue breakdown around the site where they are injected. In a relatively short experiment (5 h) this should not cause more than mild distress because the salicylate is also a very effective pain-killer and anti-inflammatory agent at these high concentrations. Over the first two hours the localised injection of salicylate is dispersed and distributed throughout the body via the blood circulation. We will conclude all our experiments within five hours of administering salicylate while its therapeutic actions are still near maximum.

The unilateral noise exposure used to induce chronic tinnitus is a non-invasive procedure , under general anaesthesia, that does not seem to be associated with any appreciable pain subsequently. During the period of general anaesthesia the animal will have reduced control over its breathing, blood pressure and temperature but external measures will be taken to compensate for this. Initially there will be a mild temporary hearing loss, but the procedure is designed to avoid producing any permanent hearing loss. Although tinnitus will develop in some of the animals, they should not find it distressing because the sound percept in tinnitus is relatively quiet (about 20 dB above hearing level) and the percept will sound like an externally generated background noise.

The behavioural testing necessary for determining the presence of tinnitus will be mildly distressing for the mice because 1) they feel threatened by having reflective markers placed on sensitive parts of the head (such as the ears), 2) they are in isolation during the testing, and 3) the presented noise pulses are designed to startle them. We hope to identify a startle movement in mice such as neck extension or tail flick that avoids sticking markers to the ears. However, during the initial experiments to compare marker position we may need up to 15 periods of sedation/anaesthesia while placing ear markers. The cumulative effect of these periods of sedation and testing may move the procedure into the moderate category. After noise exposure some animals may develop a hypersensitivity to loud sounds (hyperacusis) that is moderately distressing and can lead to flight behaviour from the startle sounds.

These animals become calm again when returned to their home cage, but they may have to be withdrawn from the study and are killed by a Schedule 1 method. This is expected to apply to less than 10% of the animals.

The subcutaneous injections of experimental drugs may cause mild and transient discomfort but compound and doses are fully characterised and no systemic effects are expected other than mild bradycardia or muscle trembling at the highest doses.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

With the salicylate animals, less than 10% are expected to enter the moderate category and none will remain in this state for more than five hours.

With the noise exposed animals no more than 10% would be expected to show moderate symptoms of distress and the rest should only be in the mild category.

With the initial studies to find the most effective position for reflective marker placement six of the mice (less than 20%) may require multiple periods of sedation that may be classed as moderate but the other mice should not exceed the mild category.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are trying to identify drugs that would be effective in alleviating tinnitus, but before beginning clinical trials we need to obtain preliminary evidence of their efficacy using animal models. As tinnitus is a conscious phenomenon and its neural basis is not properly understood we cannot currently use in vitro or computer modelling methods to test the effects of these drugs. Previous research on humans has been able to demonstrate pre-pulse inhibition (PPI) using the small muscle potentials associated with the vestigial pinna reflex and have found similar changes in tinnitus patients as we expect to see in our mice. We also know that tinnitus is a good measure of salicylate toxicity in the clinic. This reassures us that our mice models are closely analogous to human tinnitus. Currently there are still no drugs that have been approved for specifically treating tinnitus in either America or Europe and the clinical trials to date have still not found any drug that is broadly effective.

Which non-animal alternatives did you consider for use in this project?

Computer modelling of the effects of the drugs on the firing rates of nerve fibres in the cochlear nerve.

Why were they not suitable?

The cause of tinnitus is still not properly understood and the mechanisms that cause its development have still not been worked out. We do not know enough about the neural mechanisms producing tinnitus in order to model the effect of altered neuronal firing rates on its development.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Developing a behavioural test to identify tinnitus in mice using the ear-flick or other head, body or tail reflex movements

Before starting the tinnitus experiments we want to optimise the behavioural method for identifying tinnitus in Protocol 1. This will involve studying the acoustic startle response, so that we identify the method that is the most refined and reliable. We don't want to risk having to change our analysis method half way through the study as this could involve repetition of experiments or the need for larger group sizes initially. The acoustic startle involves many groups of muscles and these are recruited at different sound levels (Pantoni et al. 2020, Quantifying the acoustic startle response in mice using standard digital video. https://doi.org/10.3389/fnbeh.2020.00083). Thus, we will need to optimise the position of pairs of reflective markers that are tracked by our three cameras in 3D space. This Optitracks tracking system has not been used with the acoustic startle in mice before and we will need to try out different positions of pairs of markers to find the optimal positions that involve the mice in the least discomfort and give the most reliable indication of the startle reflex at the lowest sound levels. We will need to try attaching markers to different points on the 1) cheek, 2) pinna, 3) head at midline, 4) lateral thorax, 5) abdomen, 6) spinal column and 7) the tail. Measurements will be made of the movements of two markers relative to each other. Our aim will be to find positions for the minimum number of markers causing the least distress to the mice but that will also give a reliable measure of the reflex. Mice do not like being touched near their head and placing markers on the pinna may require transient sedation. No more than six markers would be placed on a mouse at any one time and no session would last more than 50 min. but they may be shorter than that if the mouse appears distressed. We will also need to try different marker sizes (3 or 4 mm) and different types of bedding in the chamber to minimise reflections. Because of these 9 variables and the need for quantitative data using groups of up to 6 mice (3 of each sex) we expect these initial experiments may involve up to 9 X 6 = 54 mice. Each mouse would have multiple periods (on average 10) of behavioural testing where we altered the stimulation and recording parameters. At the end of this initial testing

we would conclude the experiments (Schedule 1 method) as we would not want to expose them to tinnitus induction and testing in addition.

Once the recording parameters have been optimised, subsequent mice will go through a refined period of baseline testing in protocols 2 and 3 to determine which are suitable for attempting tinnitus induction. Mice vary in their ability to perform in this behavioural test of their initial GPIAS response and we have estimated that as many as 20% of the mice may not be suitable for behavioural testing and will fail to complete the baseline testing. This is based on our experience with guinea pigs where over 20% have been found unsuitable for making behavioural measurements. Thus, we have estimated that we will start with 246 wildtype mice even although we only expect to use 200 in producing tinnitus models. Similarly with the GA mice we expect to use 50 for preliminary testing even although we only need 40 for tinnitus models. The calculations that lead to the total of 300 animals is shown in the Action Plan Section and described below.

Development of tinnitus following salicylate

In protocol 2 we will administer salicylate alone to 4 wildtype mice (plus 2 sham injections as controls) and 20 GA mice to compare the two groups. We expect that none of the GA mice will develop tinnitus but even if a few do, the use of a group of 20 should be sufficient to show a significant effect unless the effect size is quite small (less than 10%).

Development of tinnitus following noise exposure

The numbers required for the noise exposure model are estimated based upon the incidence of tinnitus generated by noise exposure. In the mice 40 - 50% of animals are expected to develop tinnitus following noise exposure. None of the knockout mice should develop tinnitus and if any do then this would support the null hypothesis that HCN2 channels are not involved in the production of tinnitus.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We plan to carry out pilot studies to improve the reliability of the GPIAS method using wildtype mice. This preliminary work should reduce the number of mice needed in the main tinnitus experiments as there should be improved reliability for the GPIAS method when using the ear/tail flick and neck/thoracic reflexes instead of the whole body startle that has been used previously in mice.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Using externally bred animals (either commercial wildtype or from our collaborator), rather than having our own breeding colony of GA mice will greatly reduce the number of GA animals needed overall. By using mice of both sexes we will reduce the numbers of unwanted mice in our collaborator’s colony of GA mice.

We will use a vehicle and drug doses that have already been shown to be safe and effective in studies by our collaborator and the drug company.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Choice of animal

Mice are, by far, the most commonly used laboratory species and they thrive in safe, well-maintained animal units. The mouse has been the preferred species for large-scale genetic studies and there are now highly advanced methods for altering their genetic expression and changing their molecular biology that are not available in any other mammalian species. Thus, we will be using the C57Bl/6J mouse as our collaborator has developed a cre/lox knockout of the HCN2 channel in the peripheral auditory system in this strain. This strain is the most commonly used strain for genetic manipulation and our results should be widely relevant to many other laboratories. The thin skulls of mice, which have relatively little calcification, means they have an easily accessible cochlea and they have been a preferred model for studying the genetics of the auditory system for more than 30 years. Additionally, there is a large literature on the mouse auditory system and the genes controlling its function, much of which might have to be replicated if a different animal were used (contradicting reduction).

Methods for inducing tinnitus

Tinnitus can be induced rapidly and reliably by injecting a single high dose of sodium salicylate (300 or 350 mg/kg). This is a standard method for inducing tinnitus in mice and has been used with the strain that we will be using. A single injection of salicylate should cause less overall distress to the animal than noise exposure as it avoids the periods of anaesthesia and subsequent recovery as well as the gradual onset of tinnitus over six weeks. In the past we injected salicylate i.p. and this was associated with irritation and potential damage to the wall of the gut in our herbivorous guinea pigs whose digestive process is easily disturbed. The gut of the omnivorous mice should be less sensitive to the salicylate and either i.p. or s.c. injections will be made depending on which produces the least distress. Injections via the i.p. route are simpler than s.c. in small active mice that have been scruffed, but following consultation with the NVS, he recommends that we start with s.c. injections as they should be less painful. We aim to minimise any irritation to the

gut or subcutaneous tissue and lessen the risk of any distress. We will administer the HCN2 channel blocker two hours after the salicylate and then perform all the behavioural testing within the next two hours. The HCN2 channel blockers have an analgesic effect and should reduce any discomfort. This The rapid testing means that the animal can be sacrificed within five hours of the salicylate being administered and any local inflammation is minimised. Salicylate injection in mice by the s.c. route may be safer than by i.p. injection as the LD50 for i.p. injections is 500 mg/kg but for s.c. injections it is 550 mg/kg. Salicylate is absorbed by passive diffusion and rapidly distributed to most body tissues and so both routes have been shown to be effective at increasing the levels within the inner ear that we are interested in studying.

For most of the experiments, we induce chronic tinnitus via noise exposure and although this is performed under a GA the animals are allowed to recover so that we can study the behavioural changes that occur subsequently. Previous studies have used very high sound levels during the noise exposure (120 dB SPL) that causes permanent hearing loss. However, recent work in the guinea pig has shown that two consecutive periods of more moderate noise exposure a few weeks apart may be equally effective at inducing tinnitus and does not produce any significant permanent hearing loss in the audiogram. We will initially try two periods of moderate noise exposure in the mice to determine if we can induce tinnitus in about 50% of the mice without any associated hearing loss. Reducing the amount of hearing loss should also reduce subsequent stress in the animal.

Method for identifying tinnitus

The most widely used test for identifying tinnitus in rodents is the gap-induced prepulse inhibition of the acoustic startle (GPIAS). We have been successfully using a version of GPIAS, based on the ear flick reflex, in our guinea pigs for the last eight years. The ear flick reflex has a lower threshold for production than the whole body startle and so we can used lower sound levels for producing the startle that are less distressing than those used traditionally (90 dB instead of 110 dB). Groups working with mice have mainly used the whole body startle but this response habituates very quickly and after 5 repetitions is much smaller. We now want to use more robust components of the acoustic startle, based on more localised muscle twitches such as the ear flick or neck extension, that should show less habituation. We also want to measure other small body movements such as thoracic expansion and the tail flick which are part of the startle response and show little evidence of habituation. We have recently measured the sharp intake of breath that is produced by a startling pulse at the same time as the ear flick by placing an additional pair of reflective markers on either side of the thorax in guinea pigs. Our sensitive video cameras should be able to measure thoracic and tail movements in the same way in mice. Mice are naturally active and continually explore their surroundings especially if they are outside their home cage. However, this should not be a problem for us because during the analysis of the reflex movements we measure the relative position of the two pairs of markers. During the reflex response the ears move towards each other while the walls of the thorax move away from each other. In our analysis we only extract these relative movements of each pair of

markers and discard the data about absolute position. Pilot experiments performed by our previous PhD student, who is now in America have indicated that the ear flick response is a possible method for tinnitus estimation in mice but he did not use the thoracic, neck or tail markers and they may be more suitable. The reflective markers are self-adhesive and will not produce any skin irritation.

Why can't you use animals that are less sentient?

Tinnitus is a conscious perception and by definition can only be experienced while conscious. A number of mammalian models of tinnitus have been developed but although it is possible that tinnitus exists naturally in some non-mammals there is no experimental model available. The mechanisms underlying tinnitus are not fully understood and so it is not yet possible to use in vitro or computer models to adequately test the effect of drugs on tinnitus.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Induction of tinnitus by salicylate injection

High doses of salicylate can lead to irritation of the stomach or gut wall and we will be particularly careful to avoid this problem. Salicylic acid has a pH of 2.4 but we will use the sodium salt as this has a pH that is close to neutral. Part of the problem with salicylate is that it is normally injected as a hypertonic solution and this may cause transient oedema. We will initially use the sub cutaneous (s.c.) route for injections and will apply local anaesthesia ointment (e.g. Marcain, EMLA cream) to the skin beforehand if we find any evidence of swelling. Alternatively, we may use direct injections into the peritoneum as that is the standard method in mice. Animals will be carefully restrained and injections performed by trained and competent staff in accordance with LASA guidelines. We will always seek to minimise any distress to the mice during the injection of substances and will seek the advice of the NACWO or NVS. The condition of the animals will be checked frequently, typically every half hour immediately after the injection for up to two hours to check for any signs of ill-health e.g. changes in home cage behaviour or activity.

Recovery anaesthesia for noise exposure

The main risk of adverse effects are related to the anaesthesia, including remote management and monitoring required to record the auditory brainstem responses (ABRs) and for exposure to noise in one ear. By careful observation using for example a pulse oximeter and camera with monitor it is possible to check for changes in blood oxygenation or breathing rate and this should allow remedial action. Oxygen will be available both before and during anaesthesia. The duration of ABR recordings will be kept to a minimum (15 min) by only recording at a small sample of selected frequencies. After recording the post-deafening ABR a reversing agent such as atipamezole may be injected (i.m.) if appropriate and as recommended by the NVS to aid recovery. Administering a general

anaesthetic may lead to transient, mild distress due to the physical restraint or injection [or insertion of a hypodermic] needle. This will be minimised by making sure staff are fully trained and monitored for competence before making injections. We will ensure smooth and swift recovery by placing the animal in a warmed recovery cage and ensuring a good posture conducive to easy breathing and offering accessible and palatable diet.

Behavioural testing

One of the conditions sometimes associated with noise-induced tinnitus is hyperacusis which is an increased sensitivity and decreased tolerance to loud sounds. If animals experience hyperacusis then they may become very restless and repeatedly attempt to climb out of the chamber in the sound booth during testing. The first time this happens testing will be stopped and the animal returned to its home cage. If it happens three times in a row the animal will be removed from the experimental group and either used to collect brain tissue following perfusion with fixative or killed by a Schedule 1 method.

Animals are never restrained during behavioural testing and will not experience more than a mild level of stress from being placed in the test booth. They will never be left in the booth for more than an hour in any one day. While in the booth they will be kept under constant observation using three infrared cameras. The mice are briefly restrained during the placement of markers but initial experiments will try to identify a placement method and positions for the markers that will minimise this stress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All recovery experiments involving a general anaesthetic involve a risk of injury to the animal and the risks increase with the duration of the anaesthesia. Advice will be taken from the NVS and BSU Director about recent refinements that may useful. We will keep abreast of the literature for developments in animal models and will follow guidance on the NC3Rs website (https://www.nc3rs.org.uk/) as well as the Norecopa website.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will rely on guidance about improved methods from the Named persons who will provide detailed oversight and assistance with all aspects of the work. They have already been working with us over the last few years to help us improve our experimental methods using guinea pigs and this will continue with the mice. We will also consult the NC3Rs website, the website of Norecopa (https://norecopa.no/more-resources/culture-of-care), attend their meetings or training sessions where appropriate and keep informed by searching the relevant literature as well as consulting with our collaborator in Kings College London.

37. Genetic and Chemical Validation of Drug Targets in Leishmania

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

leishmaniasis, parasite, Leishmania, virulence, drugs and vaccines

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim is to characterize the key biological processes of Leishmania parasites that are required for infection and virulence. This will inform novel strategies for drug and vaccine development for leishmaniasis

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Parasitic disease of humans remains a major worldwide problem. Leishmaniasis is caused by the single-cell protozoan parasite Leishmania and is categorized as a Neglected Tropical Diseases (NTD) by the World Health Organization (WHO). This disease is prevalent in tropical and subtropical areas of the world. It causes significant morbidity and mortality amongst the world's poorest populations resulting in severe costs in both health and economic terms and draining resources that could be used to promote development. Unfortunately, treatments for leishmaniasis are inadequate as there are no effective vaccines and existing drugs are old, difficult and often expensive to administer, toxic and ineffective, with drug resistance becoming a growing problem. The development of new safe drugs, or vaccines, for the treatment of these diseases is therefore urgently needed. A better understanding of the biology of the parasite and its interaction with the host will support drug and vaccine discovery.

What outputs do you think you will see at the end of this project?

The main benefit of this project will be the knowledge gained about key biological processes affecting the virulence and pathogenicity of Leishmania which will inform our two main research areas:

1) Identification and validation of new potential drug targets that can be used by us and others to develop anti-parasitic compounds and inhibitors to, for example, protein kinase or peptidase targets.

2) Testing of potential drugs for anti-leishmanial activity and the investigation of pharmacodynamic and pharmacokinetic parameters of drugs, drug combinations and formulations to inform pre-clinical and clinical development such as rate-of-kill, drug exposure at target site (dermal skin layer for CL) and internal organs (liver, spleen and bone marrow) for VL.

Our findings will be published in academic journals and shared with the scientific community through conferences and meetings.

Who or what will benefit from these outputs, and how?

Academic scientist working on drug and vaccine discovery for leishmaniasis.

How will you look to maximise the outputs of this work?

All of these findings will be published in academic journals and further shared with the scientific community through conferences and meetings.

Species and numbers of animals expected to be used

- Mice: 2250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All the research outlined in this project will use mice as an animal model. Mice are a suitable model for these studies because many pathogens that infect humans also infect mice with similar pathogenic outcomes (e.g., Leishmania mexicana, the protozoan parasite that causes cutaneous leishmaniasis in humans). Mice can also respond to drug treatment in a similar way to humans and are thus useful for testing anti-parasite activity of new potential drugs. Research using mice has had a long history of making important contributions to the understanding of human biology and many valuable resources (such as gene-deficient mice) are available. Therefore, they enable us to make scientific advances with an increased confidence of correctly interpreting the outcomes of experiments designed to discover new treatments and therapeutics to improve human health. We will use adult mice because they have a well-developed immune system.

Typically, what will be done to an animal used in your project?

Mice will be infected with Leishmania parasites, some of which may have been genetically manipulated, and the virulence of the parasites assessed by monitoring growth of the parasite and the onset of leishmaniasis disease. The response of the animal to infection may also be evaluated, for example by taking blood samples.

After leishmaniasis has developed, the mice will be treated with potential drugs to assess whether these can cure the disease. To quantify how much pathogen is present, we can use an advanced form of imaging where a chemical is given to the infected animals and reacts with a gene in the pathogen to emit light. The administration of the chemical is done by injection and is repeated several times during the course of the infection.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the animals used in this project show no outward signs of distress or suffering. Injections produce mild discomfort and some stress associated with handling. Anaesthesia is used for restraint where needed, for example in in vivo imaging, and recovery from anaesthesia occurs within minutes.

Infections are always terminated before any severe symptoms occur and in most cases before any clinical symptoms occur. Weight loss can occur over a period of several weeks, as a result of the infection or during the administration of drugs, but will not exceed 20% body weight and in most cases is negligible. Cutaneous leishmaniasis causes skin lesions

at the site of injection that develop over several weeks, but these do not impede normal behaviour.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We do not expect that any procedure will exceed moderate severity and the majority of procedures performed under this licence (>95%) will be of mild severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our first aim is to understand and reduce the disease caused by Leishmania parasites. To do this we need to understand the mechanisms that parasites use to survive and grow and this includes necessary stages through the mammalian host (eg mouse).

In our second aim on drug discovery we often observe significant anti-leishmanial effects in vitro that don’t necessarily translate to in vivo data. This can be attributed to drug availability in the host. Doing in vivo drug assessment is the only way of evaluating whether a compound would reach and kill Leishmania in the mammalian host. There is currently much interest to better define the pharmacokinetic and pharmacodynamic properties of existing and new drugs so that we may more accurately predict which drugs are likely to be efficient in vivo.

Which non-animal alternatives did you consider for use in this project?

Growth of Leishmania in tissue culture

Why were they not suitable?

In vitro culture techniques for the growth of both Leishmania and mammalian cells have improved over the years and this allows us to do most of our work in vitro. In some cases parasite lines have recently been isolated from humans or animals and require adaptation to in vitro culture. This is often possible but in some cases not and such lines need to be continuously grown in vivo. With the increased use of in vitro culturing it has come to light that there are marked differences between strains grown in vitro and in vivo with effects

seen in, for example, virulence and drug sensitivity. Since a large part of our work relates to drug treatment and virulence we need to assess these in vivo where data may be more relevant for future drug development for treatment of human disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals based on the design of the experiments we wish to carry out and from past experience using the procedures in this application

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The experimental setup proposed in this application is already established and has been used on previous licences. Experience and data from the previous work has allowed the refinement of protocols and the reduction of animals. This has included the use of in vivo imaging to allow longitudinal assessment of parasite burden, the use of pools of bar-coded Leishmania mutants to assess the virulence of multiple parasites in one group of animals and the use of skin microdialysis to replace skin biopsy to investigate pharmacokinetic and pharmacodynamic relationship of new drugs.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Whole body non-invasive imaging is used to generate longitudinal data from small cohorts of mice

Experiments are often conducted in parallel to allow re-use of tissues/cells between experiments, reducing the number of mice needed only to supply tissues and cells

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mouse models of infection are well established for Leishmania - we are not aware of other mammalian models that can replace rodents.

Why can't you use animals that are less sentient?

Mammals are a natural host for the Leishmania parasite. We require the use of a mammalian species which contains an adaptive immune system that has clear similarities with the human immune system. We must use adult animals that have a fully developed and functioning immune system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Procedures will be refined by continual improvement and discussions with the scientists and animal technicians, NACWO and NVS to create a culture where the welfare of the animals is central to experimental design. Regular training and protocol review will ensure that any refinements will be implemented promptly

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow ARRIVE2 guidelines for reporting of experimental results. The NC3Rs and LASA web sites provide a resource of up-to-date protocols, publications, guidelines, videos and other information that enables the rapid dissemination of the most up-to-date best practices. Discussions with other scientists at conferences and within the establishment will ensure that improvements in local practices are quickly implemented. Examples of where procedures have been recently refined are the single use of needles, providing enrichment in the cages (fun tubes, wood bars), adopting tube handling, and use of animal body cues such as the grimace scale to observe pain.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly discuss refinements for animal experiments with the Named Animal Care and Welfare Officer (NACWO), named veterinary surgeon (NVS), and animal technicians. We will keep abreast of developments discussed and implemented by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) through their monthly newsletters and blogs. The animal facility holds regular forums and updates on the current best practice and latest developments.

38. Pharmacokinetics of Novel Therapeutic Agents and Disease Modification

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, central nervous system, therapy, drug movement through the body, efficacy

Animal types	Life stages
Mice	adult
Rats	embryo, pregnant, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to identify potent compounds that can be developed into novel therapeutic options for patients suffering from cancer or diseases of the central nervous system (CNS). We will assess how the novel compounds move around the body and eliminated and while doing so, how they modulate biological processes that are involved in progression of the target diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer and CNS diseases cause long-term disability or death in humans. There are many types of these diseases and specific and potent therapeutic options are desired. Many of the current therapies have limitations such as incomplete response, relapse or emergence of resistance. Options for CNS diseases, such as Alzheimer's disease, amyotrophic lateral sclerosis (ALS) and Parkinson's disease are even more limited. Discovery of novel drugs will expand the therapeutic options for patients. Work under this programme will lead to identification of novel potent drug pre-candidates that could be developed into such novel therapies.

What outputs do you think you will see at the end of this project?

Benefit from this project would be the identification of novel compounds with good ADME (absorption, distribution, metabolism, and excretion) with low toxicity, as well as the ability to change the behaviours of disease- or symptom-causing molecules within the disease type of interest. In the case of cancer- targeting compounds, we will demonstrate their efficacy in appropriate tumour models. For CNS diseases, we will generate data to support further investigation into safety and efficacy in CNS disease models conducted externally. All results will be used to evaluate, together with our clinical development team, whether the compounds studied are appropriately safe and efficacious to be considered for evaluation in human patients.

Data obtained from our work will be published, thus advancing knowledge of disease processes and drug discovery in the scientific and clinical community. Since 2002, we and our collaborators have published approximately 70 journal articles containing in vivo data in peer-reviewed scientific journals, including 10 articles in the last four years. In addition, we regularly report our findings at national and international cancer and drug-discovery conferences.

Who or what will benefit from these outputs, and how?

Short term, we will have gained knowledge of the biology of the targeted disease processes at the molecular level and how they can be modified by the novel compounds. Selection of models and their validation typically takes 3-6 months. Testing and optimising novel compounds in the chosen screening model will take a further 12-24 months. We will learn how these compounds move around the body and excreted and while doing so, whether they can modify biological processes involved in disease progression. Data generated at each step will help improve compound design. We hope to identify promising compounds to progress into further preclinical investigation. For cancer, we will be able to show efficacy in experimental cancer models.

Data generated on the promising compounds will inform clinical and preclinical investigators as to how the compounds could be used. For oncology drugs, we aim to predict which cancer types are most sensitive to the novel compounds and generate data

using models that resemble such patient populations. Using the various types of data we generate, we will be able to show the dose range and possible schedules to be used in the clinic. In order to maximise the potential benefit, we will also consider developing novel combination therapy options as well as monotherapy use, which could also impact clinical trial design. For CNS diseases, basic compound data, such as how moves around the body, biological effects and possible dose schedules will be used to engage and inform collaborators or further evaluation of the chosen compounds in the most disease-relevant models.

Data on the most promising compounds and their selection processes are also communicated to the wider scientific and clinical community. Typically, we present our findings at conferences first then as peer-reviewed journal articles. As we already have multiple projects in the in vivo testing phase, we will be publishing findings regularly (2-4 journal articles and 5-6 posters at international conferences per year).

How will you look to maximise the outputs of this work?

We collaborate closely with the technical team of the Establishment for scientific procedures. We discuss experimental approaches from both the scientific and technical aspects to maximise output from our work onsite. Their compliance team also provides us with the network to disseminate some of our findings and any refinement of procedures.

We have multiple collaborations with experts, both academic and industrial, in various disease and/or therapeutic areas. We continuously discuss the best approaches to test or use the novel compounds and how to interpret the data. Our discussions include our clinical development team to ensure that we perform the studies most relevant to progression into the clinic.

Our publication strategy includes conference presentations as well as journal manuscript. Conferences are useful means for faster dissemination of new knowledge than via journal manuscript. Unsuccessful approaches will be included in publications where possible and particularly where the drug discovery processes are described. Investigations that led to de-validation of potential drug targets, will be useful for the scientific and clinical community, therefore, considered for publication.

Species and numbers of animals expected to be used

- Mice: 24050
- Rats: 2290

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice and rats will be used because they are the lowest vertebrates that have well-characterised models to study drug exposure patterns and efficacy. They share sufficient anatomy and physiology with humans to be useful for the prediction of how effective the potential therapeutic compounds are in the clinic.

The majority of our studies will use adult mice, as they have been extensively used in initial evaluation of novel therapeutic compounds and detailed standard protocols are available in the literature and in- house. Many strains are also available as hosts for implantation human tumour implantation, allowing investigation of human diseases such as cancer. Very young animals will generally not be suitable for our studies involving compound dosing due to the study duration, compound administration and sample requirements.

This programme also includes the use of animals for providing fresh tissues and organs for testing compounds in the laboratory, where no suitable commercially-available cell lines are available or the use of stem cell-based systems is inadequate. Neuron preparation is more successful from young animals than from adults, therefore, the use of rat embryos and newborns for primary neuron preparation is included.

Typically, what will be done to an animal used in your project?

This programme is designed to assist drug candidate selection and development, through a series of experiments using mice and rats. Regulated procedures include substance administration, blood sampling, minor surgery, subcutaneous tumour cell inoculation and tissue collection under non- recovery anaesthesia. Each study will use one or more of these techniques.

In the initial experiments, we aim to investigate if the novel compound has drug-like properties, in other words, assess how the novel compound moves through the body to predict whether they are likely to cause desired biological effects. Animals will receive one dose of a compound at a low level and blood samples taken at various timepoints. Compound levels are measured in the blood and tissue samples of interest, which are collected after the animals are humanly killed. This will be followed by a confirmatory study using a higher dose levels or after a short period of repeat dosing, and assess whether the compound treatment results in the desired biological effects, such as inhibition of disease-driving proteins.

The subsequent study will test the safety of the predicted therapeutic dose schedule in a small number of animals. Animals will receive repeat compound administration for 1-2 weeks. Bodyweights will be measured and animals monitored regularly for any signs of ill health. The animals may have blood samples collected during the study to confirm the predicted compound exposure.

In studies to assess the efficacy of novel anti-tumour compounds, mice will receive tumour cell implantation under their skin on their flanks. Once the tumour growth is confirmed, a

compound solution or its vehicle will be administered to the animals at a dose schedule that was found to be safe in the previous study. Treatment period is typically 2-4 weeks. Most animals will receive one tumour implantation in their life time. Where we wish to test multiple tumour models, two types of tumour cells with similar growth rates may be implanted on opposite flanks. In all cases, tumour volumes are closely followed and animals humanly killed before the tumours start to impact animal health. Mice that have been cured by the treatment may be challenged by an additional tumour cell injection to test whether any long-term anti-tumour immunity has been induced. We will also assess combination therapies, where more than one treatment are given, which may improve the efficacy. A small number of animals will receive minor surgery to implant tumours or hormone-releasing pellets under their skin.

All animals are intended to be group-housed as isolation is known to cause stress in animals. In some cases, social housing is impossible on the welfare ground (due to aggression, surgical procedures or when we need to collect urine in certain study types). The mice will be housed on their own with increased environmental enrichment where appropriate.

We will investigate compound elimination and excretion of the most promising compounds using bile duct cannulated rats. The cannulation are done externally before arriving at the facility. Animals are given compound solution and bile and urine collected.

What are the expected impacts and/or adverse effects for the animals during your project?

Procedures for substance administration will cause transient pain during and after IV, IP or SC injection and no lasting discomfort.

In-life blood sampling through puncture of the superficial veins can result in transient bruising at the sampling site. Any bruising and swelling from repeat blood sampling may last up to 24 hours.

Administration of novel compounds may cause some bodyweight loss and other ill effects, such as diarrhoea, rashes and general signs of discomfort, similar to the effects experienced by cancer patients undergoing therapy. These typically occur gradually over 4-10 days. These can often be managed by modifying dose levels and frequencies and by providing diet supplements.

Experimental tumour growing under the skin on the flanks of animals cause little adverse effects. We have a set carefully-chosen humane endpoint so the studies are terminated before the tumours can impact the animal health. Tumours growing too close to limbs or on the abdomen could hinder movement or lead to abrasion. A few tumour models cause unintentional bodyweight loss. Tumours from some cell lines are prone to grow into the skin and develop lesions on the skin over the tumour over time. Animals showing these will be excluded from compound treatment studies. There are a few exceptions, such as when

treatments are known to cause inflammation within tumours which can lead to development of dry scabs before tumours shrinkage.

A small number of animals that received minor surgery will be given pain relief therefore no long-term discomfort is expected. Animals that have have received surgery before arriving at the facility are likely to feel some general discomfort.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Of the total mice in this programme, 90% of the total mice used will experience mild severity. They will receive compound injection or blood sampling on one or repeated occasions. Up to 30% of the mice on tolerability studies (2% of total) and 10% of the mice on subsequent tumour studies (6% of total) will experience moderate severity. This is where the first investigation of the effects of repeat-dosing of novel compounds at potentially therapeutic doses are performed. We also test combination therapies, where the toxicity is also likely to be enhanced. Approximately 2% of the mice will experience only procedures under non-recovery anaesthesia.

Of the total rats, we estimate that 85-90% will experience mild severity. They will receive compound injections and needle-stick for blood sampling. 5-10% of the animals will experience only procedures under non-recovery anaesthesia. Up to 5% of rats will have had surgery before arriving at the facility.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

For Objective 1, we use animals to provide fresh tissue samples to study neurons and other cell types present in the central nervous system. The number of cell lines that can be used is limited.

For Objectives 2 and 3, we need to use animals to show that the novel compounds are able to move through the body and reach the target diseased tissue to act on, while having minimum toxicity.

Currently, there is no non-animal system that sufficiently mimics the entire body system.

Which non-animal alternatives did you consider for use in this project?

For Objective 1, we are setting up human stem-cell system which can provide neurons and other cell types of human origin for compound testing in the laboratory.

For Objectives 2 and 3, we have considered organs-on-chip and liver organoid assays for pharmacokinetics, tumour organoid assays for drug efficacy and mathematical modeling to predict compound movement through the body and its bioilogical effects.

Why were they not suitable?

For Objective 1, human stem-cell systems are currently being established for future use and not yet ready for regular use in-house.

For Objectives 2 and 3, although useful for testing a few aspects of compound properties, organoid systems cannot sufficiently mimic compound passage through the whole body. We use mathematical modelling to predict relationship between compound exposure and biological effects in animals. This still requires an initial set of data generated in animals to create the model, which can then be applied to later compounds. A confirmatory study in animals is needed to before a compound can be recommended for further investigations including clinical trials.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the types of studies required for each of our internal projects, and compared against animal use over the past 5 years.

We currently have 8 active cancer and CNS disease projects at different phases. These phases include early exploratory, compound optimisation and clinical translational. The types and the volume of animal work vary depending on the stages. As the projects advance, new projects will be added, therefore, the general volume of the animal work remains constant. We estimate that we will be conducting up to 40 rat ADME studies, 100 mouse ADME studies and 90 biological studies (tolerated dose schedule finding, study of molecular changes, efficacy demonstration) in mice per year. In addition to this, we are including a limited number of mice and rats for organ collection protocol which will be used to provide fresh tissue for assays in the laboratories or as pilot studies for future biology studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have been conducting animals experiments for drug discovery programme since 2002. This includes a range of study designs and output variability for each type of experiment. The study designs have been optimised over the years and routine work standardised in such a way that they allow comparisons to historical data, therefore, most of the studies we conduct under this PPL will be based on these.

Compound exposure studies are designed to allow analysis using specialised ADME software. We will need a minimum of 3 replicates per data point to generate robust results. We will use repeat blood sampling, which reduces the animal requirement by up to 60% in mice and up to 85 % in rats.

Confirmation of tolerability at a predicted therapeutic doses is typically sufficient with 3 mice. The study duration is typically 7-12 days to ensure capturing of both early- and late-onset effects. We predict the the safe therapeutic dose range from tumour- and normal cell potency data together with ADME studies. We typically start by testing the highest predicted therapeutic dose in 3 animals. In majority of the novel compounds we design, generate and test, the originally chosen dose schedule causes no notable ill health. We use a staggered start approach to minimise the number of animals used. If the first animal shows immediate signs of toxicity, the dose can be lowered for the second animal or the study paused for review. In such cases, we may use a total of up to 6 animals.

Most efficacy studies with tumour-bearing animals will need 6-8 mice per treatment to show difference between treated and control-treated. Statistical analysis was performed on several recent data to confirm this. If the treatment is predicted to be highly effective or variability in tumour growth is particularly small, we can reduce the group size.

For protein or gene expression analysis, we have used 3-6 mice per timepoint per treatment. We have reviewed the design and results in recent studies to show that sample size of 3 is generally sufficient to show robust trends in oncology studies. For CNS diseases, where the disease-related signals often are smaller than in cancer, we have found group size of 4-6 is typically necessary.

For investigation of a new potential drug target, we study the literature to understand the possible range and duration of biological effects (differences between the level in diseased and normal tissue) needed to change the course of disease, and use them to modify our study designs. Small-scale pilot studies will be conducted to assess the suitability of the model and further optimise the study design, such as group size, study period and sampling timepoints.

Mathematical modelling is used to predict the relationship between compound exposure and biological effects. An initial set of data generated in animals is used to create the

model, which is then be applied to later compounds. This can remove the need for some studies and ensure only the compounds showing promise are tested further in animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For initial testing of novel compounds, we will use male mice, which are generally larger and able to provide higher volume of blood samples. Compared to using females with reduced blood sampling frequency, this can reduce the animal use by up to 40% in the mouse ADME studies.

For determination of biological effects of compound, pilot studies will be performed to understand the variability of the readout, which will be used to adjust the group size in the subsequent studies.

Experimental data will be used to build a mathematical model to predict the effects using output of low- dose ADME studies, reducing the number of compounds tested in subsequent studies.

In subcutaneous tumour studies, we typically plan multiple studies (efficacy, therapeutic-dose ADME or tolerated dose schedule confirmation) together. From a batch of tumour-bearing mice, those with a certain range of tumours (typically 60 - 250 mm3, 70-80% of the mice) are selected for an efficacy study. Those with tumours above or below this size range or that are awkwardly-shaped and difficult to measure will be used in other types of studies. Compared to preparing tumour-bearing animals for individual study types separately, the usage of tumour-bearing mice is reduced by up to 30%.

We plan to use mice that have failed to develop tumours after subcutaneous cell injection in therapeutic-dose ADME studies or tolerated dose schedule confirmation studies. This reduces the usage of new animals in such studies by up to 20%.

At the end of studies with tumour-bearing animals, tumours and other tissues may be collected from vehicle-treated animals and stored for future use. They are used for the investigation for the same project or future projects.

Treated animals in efficacy studies or tolerability confirmation studies may be blood-sampled to confirm compound exposure and allow correlation to the observed biological effects in the same animals. This removes the need for dedicated repeat-dose ADME studies in non-tumour-bearing animals.

Some cancer models may also be utilised for investigation of CNS diseases if the target protein is the same. CNS disease models are highly variable, requiring a high number of animals, a long study duration and may involve surgery or injecting substances into the brain. We do not propose to conduct such studies under this programme but by external collaborators only with the most promising compounds. We would like to conduct the initial compound selection in a more reproducible and aless stressful cancer model, adapted to

also investigate compound levels in the brain, to reduce the overall usage of animals for the CNS disease project.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice and rats will be used during this project. They are the lowest vertebrates that share similar physiology with humans and many well-characterised models in these are available for both ADME and efficacy studies.

The majority of our studies will use mice, as they have been extensively used in preclinical evaluation of novel therapeutic compounds and detailed standard protocols are available in the literature and in- house. Many mouse strains are also available as hosts for human cancer implantation, allowing the investigation of human diseases.

Equipment for oral dosing will be chosen according to the species and the size of the animals. In order to administer a specific dose of a compound, we use a syringe attached to a curved blunt needle with a bulb at the end for mice or a flexible tubing for rats. Such needle or a tubing is gently inserted through the mouth into the stomach where the compound in liquid preparation is released. This can be stressful for the animals and without good skills, it can cause damage to oesophagus or trachea. Dipping gavage needles in sugar syrup can mask the taste of some compounds and act as lubricant, generally help reduce animal stress.

Wild-type mice and rats will be used for initial ADME determination. Animals will receive compound administration and serial blood samples will be taken from superficial blood vessels. In mice, we collect blood from a surface vein of the lower hind leg as it is visible and easy to access and requires no anaesthesia or pre-warming which are stressful to the animals. Each mouse is typically sampled 2-3 times but this may be increased if a lower blood volume per sample is required or larger mice are used. For rats, we are able to collect blood samples from more timepoints because of their body size. We use temporary tail vein cannulation which avoids venepuncture and pre-warming each time a blood sample is collected. Temporary tail vein cannulation is quick to prepare and also causes less discomfort than deep-vein cannulation that requires surgery under anaesthesia. These studies typically last no more than 24 hours and animals experience pain once at the time of cannula insertion and only transient discomfort during each blood sampling procedure.

Other studies that require multiple dosing of compounds at higher, potentially therapeutic levels will be monitored closely. Tolerability studies will use mice of the same strain (or the same background strain) as the subsequent disease models in order to reduce variability and for better prediction of safety. In some cases, animals that have undergone tumour cell injection will be used to further improve predictability.

Experimental tumour models induced by injection of cancer cell lines under the skin are extensively used in oncology research. These are quick to prepare, allow the use of cell lines that have been used in laboratories, and cause little discomfort in mice as long as the tumours develop in the flanks.

Tumours are measured externally, and therefore, do not require procedures associated with measuring tumours that develop internally.

Cancer models can be a useful tool for investigating certain types of CNS diseases as they can be caused by deregulation of the same proteins. These proteins may only be detectable in diseased brain or other tissues, making investigation in healthy wild-type animals impossible. CNS disease models generally require a long study period with highly variable results, thus requiring high numbers of animals. Animals are likely to suffer gradual and long-term disease-related symptoms. Instead, a cancer model expressing the target protein offers a less invasive, relatively quick and more predictable method for initial assessment of biological effects of novel compounds in animals. The correlation between compound levels and the biological effects in the tumour can also be applied to brain concentration to estimate the possible level of biological activity in the brain. Promising compounds can then be selected and tested in further disease models.

Why can’t you use animals that are less sentient?

Due to the duration of the compound-dosing studies (typically 24h for ADME, longer for other types of studies), it is not possible to complete all procedures under non-recovery anaesthesia. Use of immature mice or rats will not be be able to meet the requirements for repeat blood sampling or tumour induction.

Demonstrating compound effects on tumours of human origin is necessary for drug discovery. The use of less sentient species for cancer models, such as chick embryo models and the use of zebrafish, have been reported. However, they cannot mimic the compound movement through the mammalian body, therefore, insufficient for drug discovery programmes.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We monitor and record animal status in all experiments, including relatively short ADME studies. We have developed study type-specific record sheet in which observations are

recorded at regular intervals so any progressive issues can be found easily and be dealt with quickly.

Tumour-bearing animals will be monitored regularly. For the majority of the models used, tumours double in size every 3-7 days and we have found measuring tumours 2-3 times a week is sufficient. We will increase the measuring frequency when using tumour models that double every 1-2 days.

The surgical procedures included in this project are limited to minor implantation of tumour fragments or slow-drug delivery devices under the skin. Pain relief will be used to alleviate transient pain and discomfort in animals that have undergone surgery. We will purchase all surgically-cannulated animals from reputable commercial sources and we will monitor the surgery wounds and animal health.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Diehl KH, et al., (2001) A good practice guide to the administration of substances and removal of blood, including routes and volumes. J Appl Toxicol, 21:15.

Workman W, et al., (2010) Guidelines for the welfare and use of animals in cancer research. Br J Cancer, 102, 1555.

LASA 2017 Guiding principles for preparing for and undertaking aseptic Surgery. A report by the LASA Education, Training and Ethics section. (E Lilley and M. Berdoy eds.)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We maintain a close working relationship with all facility personnel involved in ensuring our adherence to the principles of 3Rs. This ranges from the facility manager and skilled technical staff, veterinary surgeons and more. We receive regular technical and training review. We will implement improvements according to new guidelines or recommendations as they are published, and undertake 3Rs assessments regularly.

We will also use our network of similar-minded investigators, including academic and industrial collaborators, to obtain further information.

39. Glucocorticoids: Breaking the Link Between Obesity and Insulin Resistance

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Glucocorticoids, Obesity, Insulin resistance, Diabetes

Animal types	Life stages
Pigs	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to understand the role of glucocorticoids (stress hormones) in the development of complications associated with obesity.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Over 650 million people worldwide are obese and 18 million die annually from its complications including diabetes and cardiovascular disease. In some people with obesity the hormone responsible for controlling blood sugar levels (insulin) stops working as efficiently, the organs in the body become resistant to the effects of insulin, this is known as insulin resistance. In the face of insulin resistance the pancreas produces more and more insulin and this can lead to pancreatic exhaustion (diabetes) and cardiovascular disease. How obesity results in these serious complications in some, but not all people, is unclear. In some diseases, such as Cushing's disease, stress hormones are very elevated and there is a clear link between these hormones and the development of insulin resistance/diabetes. In obesity, however, where there is not an obvious increase in stress hormones in the blood, the situation appears more complicated. Stress hormones are processed by organs in the body such as the liver, fat tissue and muscle. This project aims to understand the role of stress hormones in the development of complications associated with obesity, determine which organ in the body is most important in determining this risk and uncover new genes responsible for complications in people with obesity.

What outputs do you think you will see at the end of this project?

This project will help us understand how stress hormones contribute to obesity complications and therefore help identify people most at risk and develop new targeted treatments. Specifically we hope to determine which organ in the body plays the biggest role in the development of obesity complications like diabetes and identify new genes which are important in determining whether a person is more at risk of obesity complications.

Expected outputs: publications, conference abstracts and seminar presentations, raw data such as sequencing and imaging data sets. Research outputs will be made available through the research outputs portal, PURE. In addition datasets will be deposited in a data share repository.

Who or what will benefit from these outputs, and how?

Over 650 million people in the world are obese and a great proportion of these develop complications such as diabetes and cardiovascular disease. A new understanding of the underlying causes of obesity complications will potentially influence policy or strategic guidelines for healthcare professionals managing people with obesity. It will help them make the most appropriate management decisions and therefore ultimately benefit the patients themselves in terms of improved quality of life and increased life-expectancy. These outcomes are likely to be relatively long-term in their realisation but they will be globally applicable. These outcomes also apply to species such as horses, dogs and cats in whom obesity and its complications are a growing concern. In addition we will increase our knowledge and understanding of glucocorticoids in pigs which could inform work to improve the health and welfare of animals in commercial pork production.

Successful completion of the project will improve our understanding of the interaction between obesity, stress hormones and metabolic risk. This may lead to identification of therapeutic targets that will have an impact on animal and human health. For example, determining the contribution of the liver to diabetes development could lead to liver-specific targets or the re-purposing of drugs already known to act on liver enzymes. Other short-term component benefits include the optimization of experimental methods such as use of habituation and placement of vascular access ports that will offer refinement of existing approaches employed in animal research.

Early capture of intellectual property arising out of hypothesis-led research could assist in securing a competitive advantage for research and commercial activity arising out of the UK, and attract research and development investment from global business.

How will you look to maximise the outputs of this work?

In this project we will be collaborating with other scientists in Spain and Denmark to maximise the value of our research and increase its impact and likelihood of success. We will store all samples collected so that they may be used in future work following appropriate ethical review.

We will publish our findings and our research methods, positive or negative, in peer reviewed journals and present them at international scientific conferences in a timely manner. We will also disseminate information through public engagement events such as public lectures and science festivals.

Species and numbers of animals expected to be used

- Pigs: 60

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In this project we are using an adult pig model of obesity. Pigs have very similar stress hormones to humans and, like humans, when they become obese some, but not all, develop complications such as diabetes. In this study, I will compare the stress hormones and genetics of pigs at high risk of complications and those at low risk to understand how they differ. Using adult pigs we can most closely replicate the development of obesity in adult humans and we can trace the changes through obesity development. The hypothalamic-pituitary-adrenal axis, the main determinant of circulating stress hormones, undergoes minimal changes once an animal reaches adulthood and we can therefore better assess any changes we see as a result of the dietary intervention.

Typically, what will be done to an animal used in your project?

We will use two breeds of pig, one is a fast growing lean breed which is relatively resistant

to the effects of a high-calorie feeding and the other is a slow growing fatty pig which is prone to developing complications when fed a high-calorie diet.

We are studying stress hormones and therefore we will spend a considerable amount of time (2- 3months) before the start of the experiment acclimatising and habituating the animals to handling so that we can examine them, weigh them and take samples without them becoming stressed. We will take samples such as blood and urine at the start of the experiment and while they are being fed a high-calorie diet for 3 months, we will measure stress hormones in these samples to understand what happens in the different breeds of pig. The animals will also be anaesthetised and have abdominal surgery before and during the high-calorie diet. During surgery we will take small samples from the liver, fat tissue and muscle so that we can determine the amount of time of stress hormones in each tissue. We will then give the animal an infusion of stress hormone and we will take samples of blood crossing the liver, fat tissue and muscle over the course of several hours. From these samples we can determine the contribution of each of the organs to stress hormone processing. A group of each breed will also receive a drug which blocks the effects of stress hormones to determine how this affects the risk of developing obesity complications such as insulin resistance.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals will undergo procedures that may involve injections and surgery after which they may experience moderate discomfort for a short period of time (1-2 days) but every animal will receive pain relief as specified by a veterinarian. We will be habituating and training the animals for a period of time prior to commencement of the study using positive reinforcement so that any interaction with humans is as positive and stress-free as possible. The animals will be kept together in groups with access to significant enrichment. Based on our experience and the experience of our collaborators we do not anticipate any adverse side affects from feeding a high calorie diet.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The overall severity of this project is moderate because the 90% of the animals will be anaesthetised and have an abdominal incision and tissue samples taken.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In this study we aim to determine the relationships between regulation of stress hormones and the development of insulin resistance and diabetes. This is incredibly challenging to do in humans because studies are often based on single observations or limited in the degree of intervention that is ethically/practically possible. Most studies compare obese and lean states in different individuals at a single timepoint because of the difficulties of studies over time, and there are very few suitable measures which accurately reflect stress hormones for use in genetic studies.

Which non-animal alternatives did you consider for use in this project?

At the moment there is no suitable non-animal alternative for addressing the aims of this project. Cell models have been used to determine the individual effects of stress hormones on certain parameters but they often do not reflect findings in humans or animals because they are too much of an oversimplification of the system. We have done preliminary analysis using human genetic datasets to guide our questions.

Why were they not suitable?

The regulation of stress hormones is incredibly complex and interlinked, with each organ playing a slightly different role. A whole organism approach is therefore essential to unpick the complexities of the system which cannot be replicated in a cell model. There is insufficient data available in humans to rely solely on a genetic studies to answer the questions.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animals used in this study will be adult, females of the same age from two breeds. Once a pig reaches adulthood the hypothalamic-pituitary-adrenal axis which controls blood stress hormones levels, does not change significantly and so animals can act as their own

controls when we change the diet. This reduces the number of animals we need to use and improves the data quality. The data from each animal will be compared to the measures at the start of the experiment prior to starting high-calorie feeding. The number of animals is estimated based on pilot data in both breeds and our experience in longitudinal studies of obesity.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The NC3Rs experimental design tool was used to inform our study design as well as consultation with statisticians and collaborators with experience of porcine models of obesity and stress hormone modelling. The experimental approach has been developed to allow acquisition of multiple types of data (blood, tissue, imaging etc) at multiple time points, maximising the amount and quality of data acquired from each animal.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The longitudinal element of this project includes a pilot phase of a small number of pigs (n=4) to ensure the feasibility of each experimental phase and to allow us to refine any techniques. Sample sizes will be reviewed at the end of the pilot phase. Each animal will act as their own control with data collection at baseline and then during the dietary intervention; this reduces the numbers of animals required but maintains the power of the study. The studies are designed to acquire multiple types of data at each time point, maximising the information that can be gained from each animal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use pigs in this project because of their similarities to humans in genetics, physiology and size particularly with respect to stress hormone biology. My preliminary data show their stress hormone profiles are more similar to humans than rodents or other large animals. We will use blood sampling over time and then intermittent sampling of tissues. These methods are used in humans to accurately study processing of stress hormones. Pigs are suitable for experimental-medicine interventions and longitudinal sampling. Prior to entering the study the animals will be habituated to handling so that they

stand, with minimal restraint, for procedures/sampling so that unstressed samples can be obtained which is essential to achieve the objectives of the project. We will use vascular access ports as much as possible so that blood sampling via a subcutaneous port can be conducted with minimal restraint of the animal . The animals will be anaesthetised for any prolonged procedures so as to minimise handling and stress to the animals. The animals will be given peri-operative analgesia for any invasive procedures and will be monitored closely by technicians experienced in pig handling and assessment. We will use high-calorie feeding to study obesity development in pigs, the diet is designed by experts in pig nutrition to ensure palatability. The study will be conducted in a specialised facility staffed by experts in large animal handling and experimentation, anaesthesia and surgery.

Why can’t you use animals that are less sentient?

Rodent models of glucocorticoid metabolism are fundamentally flawed in the context of translation to humans and other mammals because the primary stress hormone of high mammals, cortisol, is not present in rodents. As such any study of glucocorticoid metabolism relies on conjecture and assumption. The enzyme 11β-hydroxysteroid dehydrogenase type 1, for example, showed promise as a link between glucocorticoids and obesity but its therapeutic potential has not been realised, because of difficulties translating findings from rodents to humans. Its inhibition results in compensation predominantly by the hypothalamic-pituitary-adrenal axis which could not be fully characterised in rodents. In addition the questions that are always left unanswered in rodent models of obesity or genetic manipulation of glucocorticoid regulating pathways are those of causality because it is almost impossible to sample blood or tissue from the same animal at baseline and then longitudinally during an intervention. Other models such as Zebrafish and drosophila are also unsuitable for similar reasons and in fish there is rarely a metabolic phenotype recognised in obesity models.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

This study is centred on glucocorticoids, stress hormones, as such it is essential that the animals are minimally stressed throughout the experiment. The animals will have access to enrichment (straw, toys etc) throughout the experiment and kept in stable social groups. Before animals enter the experiment for either protocol 1 or 2 they will undergo a period (up to 4 months) of training (using positive reinforcement) to habituate them to human interaction, movement around the facility, standing for sampling and weighing, temporary separation from their group, examination of laparotomy sites and administration of substances via a dosing syringe.

Vascular access ports will be placed early on in the experiment so that blood samples can be obtained via a subcutaneous port rather than repeated venepuncture, this reduces the amount of handling and restraint required and the risk of haemorrhage.

Procedures requiring the animal to be restrained for any length of time will be conducted under general anaesthesia in order to minimise stress. Animals undergoing a laparotomy will receive antibiotics and analgesia routinely.

The study will be conducted in a specialised facility staffed by experts in large animal handling and experimentation with access to state-of-the-art facilities for housing and experimental work.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have designed the experiments following the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE). We used the 3Rs experimental design tool to inform our experimental design.

The housing of animals will follow the guidelines on enrichment (van de Weerd and Day 2009) and the habituation and enrichment methods used will be reported when we publish our study findings.

For venepuncture we will adhere to the published guidelines from the NC3Rs. https://www.nc3rs.org.uk/3rs-resources/blood-sampling/blood-sampling-pig. We will also followed published best practice for the oral glucose tolerance test (Manell et al 2016, 2021).

We will report our findings in accordance to the ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The budget of the project includes ongoing 3Rs training for the animal technicians and researchers involved. We will attend and present our findings at the local 3Rs conference. We subscribe to the NC3Rs newsletter and individuals in the project team are members of the Laboratory Animals Veterinary Association, the Laboratory Animal Science Association and the Large Animal Research Network, attending meetings and staying up to date with developments. We will ensure that in all reporting of the study we include our habituation protocol and details of enrichment for scrutiny by reviewers.

40. Senescence in Early Tumorigenesis & Age Associated Chronic Degenerative Disorders

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

senescence, hyperplasia, tumorigenesis, aging, degenerative disorders

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To ascertain the role of cellular senescence and its effector proteins/pathways in early tumorigenesis (the production or formation of a tumour) & age associated chronic degenerative disorders.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is a leading cause of death worldwide. Efforts devoted to cancer research over the last decades have been formidable. For instance, a major breakthrough came from the human genome project which has enabled us to know that of the 20,000 or so genes of the human genome, only a handful of usual suspects are most prevalent in tumours. However, it has been extremely challenging, for instance, to move beyond the genetic drivers of cancer to how they lead to the cellular hallmarks of cancer: self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis (cancer that spreads from where it started to a distant part of the body), limitless replicative potential, sustained angiogenesis (blood vessel growth), and evasion of apoptosis (cell death).

Harnessing the power of genetic sequencing to study tumorigenesis in humans has revealed sub-types of cancers, again featuring the usual suspects but it also reveals a mess, cancer cell genomes are often fragmented and duplicated. It is very hard to determine, from the kinds of tumours with which human patients present at the clinic, which events have driven tumorigenesis (the production or formation of a tumour) and which have merely come along for the ride. Studying tumorigenesis in animal models allows us to study much earlier stages of the disease, even the first few days of 'cancer'. We believe that by studying these processes in mice we will be better able to understand cancer and also perhaps come up with better tools for early diagnosis, as well as therapeutic interventions.

Our tissues are constantly rejuvenating themselves, the surface of our skin is effectively replaced every three weeks and the surface of our intestines even faster still. This is thought to be to combat the harshness of our environment. At a more fundamental level our cells turn over their protein complement rapidly too, through processes such as autophagy (self eating, akin to cellular recycling). However, if cells do accumulate damage e.g. through exposure of deeper layers of the skin to ultraviolet (UV) radiation from the sun or even through mistakes made during the natural cell division that takes place to replace all these cells we shed, they may undergo a defence mechanism that results in a permanent stop of the division cycle: senescence. Studies in mice have relatively recently shown that if these senescent cells are removed then symptoms of aging can be reversed and the mice rejuvenate. The lifespan of these mice was overall unaffected but they lived a much healthier life, free from age associated diseases such as e.g. osteoarthritis. Removal of these senescent cells also reduced the numbers of mice which died of cancer.

In some tissues, senescent cells can be eliminated naturally by the immune system. However, if senescent cells persist they can accumulate forming premalignant lesions or tumours that the immune system is not able to eliminate. Senescent cells secrete a complex cocktail of inflammatory and tumour-producing factors in the surrounding tissue, which may also interfere with the immune system’s clearance and allow damage cells to

persist. We now have strong evidence, including from mouse studies, of a causal role between senescent cells and the origins of cancer.

What outputs do you think you will see at the end of this project?

Knowledge and collaboration

The primary output of this programme of work will be to increase knowledge of:

1. The different sub-types of senescence that exist within the body, rather than the senescence that occurs in cells in a petri dish. We aim to make a gene expression 'atlas' of the different types of senescence that occur in the different organs and tissues of the body across an animal's lifespan.

2. The genetic and epigenetic (additions to DNA, e.g. methylation) alterations that occur during ageing and age-reversal and whether that senescence response changes with age.

3. The interactions of senescent cells with the immune system and how that affects the clearance of senescent cells, potentially avoiding tumorigenesis.

Newly developed knowledge will be disseminated through:

1. Publication of results in Open Access, peer-reviewed, high impact scientific journals and regular presentation of data at national and international meetings.

2. Dissemination of study data through open access repositories such as GEO datasets for the genomic data.

3. Fostering collaboration and sharing knowledge between the collaborating centres.

Who or what will benefit from these outputs, and how?

This project will be of direct benefit to researchers working in the fields of senescence, aging and age- related disorders as well as epigenetics. Since our mouse model of premature aging uses autophagy perturbation, this will also be of benefit to researchers in the field of autophagy.

Developing methodologies, and mouse models

1. Publication of methods and technologies in Open Access, peer-reviewed scientific journals or online. Where methods are considered to be particularly innovative or novel publication in a methods-focused peer-reviewed scientific journal will be considered (e.g. Nature Protocols / Methods).

2. Any new mouse models will be shared with research communities upon publication or under a collaborative basis prior to publication.

How will you look to maximise the outputs of this work?

We aim to publish our new results (i.e. scientific findings, as well as the methodologies mentioned above) in Open Access, peer-reviewed, high impact scientific journals and will regularly present our data at high-profile meetings. Genomic data will be disseminated through open access repositories such as GEO datasets. Any new mouse models will be available to the research community.

Communication of significant findings will be enhanced with support from dedicated PR teams, in addition to social media platforms, such as Twitter.

Our location benefits from a thriving biotech environment from small-medium enterprise (SME) to large pharmaceutical companies. We will look to leverage the results of this study and foster academic- industrial links with segments of this community to accelerate translation into the clinic and society.

Species and numbers of animals expected to be used

- Mice: 12000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the predominant animal used in cancer research and are also one of the most prevalent organisms used in genetic research. Laboratory strains of mice have been highly inbred over the last century, to the point where the mice within a strain are nearly genetically identical allowing for experiments to be standardised and thus highly reproducible. Crucially, there are large repositories of genetically modified mice available. Key genes involved in the development of cancer have been genetically engineered so that their cancer associated forms can be expressed, at will, by the researcher, even simply by changing the mouse's diet. Together these innovations make mice the ideal animal model for research into the development of cancer.

In terms of our choice of life stage. Although there are juvenile forms of cancer, and our colleagues study these, cancer is predominantly a disease which affects adults, and indeed the likelihood of getting cancer increases with age, and we will nearly always be using adult mice in our research.

Typically, what will be done to an animal used in your project?

We will use a number of ways of causing senescence and/or tumorigenesis in our mice for our studies. For instance inhalation of genetically modified viruses by the mice will lead to

the development of tumours within the lungs. Also we inject plasmid DNA into the tail veins of our mice which leads to the development of liver tumours. We are however interested in the very early stages of tumour development, the first few days to weeks, and as such the majority of the mice we study will not go on to develop tumours, but rather at most hyperplasia (an increase in the number of cells in an organ or tissue).

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of our mice will be culled days to weeks after e.g. virus administration to the lung or plasmid DNA injection to target the liver. This will allow us to study the appearance, and immune- system mediated removal, of senescent cells in the first few days after the delivery of an oncogenic (cancer causing) mutant gene. Thus the adverse affects will originate solely from the those associated with the techniques of viral or plasmid delivery themselves.

Occasionally longer-term experiments will be necessary, where the rate of tumour development is an important end-point. We require the mice to develop measurable tumours to, for instance, allow sufficient biological material to be obtained for analysis. Where possible tumour growth will be tracked using imaging, which will allow us to identify and follow the tumour, without the need for emergent clinical signs in most cases.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice which undergo, for instance, hydrodynamic tail vein injection and/or develop tumours are deemed to have undergone a moderate severity of suffering. Based on previous usage we expect around a third (33%) of animals will fall within the moderate category. As stated above, we are interested in studying the very early stages in cancer development, the first few days to weeks. Hence, we will only need to leave mice for longer, to develop tumours, in a small proportion of our experiments.

The breeding of genetically altered (GA) animals will produce surplus mice not bearing the required alleles, as determined by Mendelian genetic ratios, and as such we expect around 59% of our mice to undergo no discernible suffering at all (techniques we use herein, such as viral delivery to the lungs and hydrodynamic tail vein injection, help to combat the wastage resulting from the breeding of GA mice). We expect the remaining mice will fall either into the mild severity category (7.6%) or the severe severity category (0.4%).

What will happen to animals at the end of this project?

- Killed

- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The interactions of cells with other cells within a tissue are too complex to recapitulate in a culture dish; for this reason it is essential to carry out some experiments on animals.

Which non-animal alternatives did you consider for use in this project?

Alternatives to animals in cancer research chiefly come down to

(a) cells grown in petri dishes, alone or in co-culture

(b) cells cultured in organoids

(c) cells cultured in tissue slices

Why were they not suitable?

With regards to (a) not all the cells of the body can be grown successfully in petri dishes. Cells of the liver for instance will either die or de-differentiate (cease to function like liver cells) for unknown reasons (presumably a lack of growth factors) once taken out of the environment of the body. Those cells that most readily grow on plastic are cancer cells, however by the time they have become a fully blown cancer cell it is almost too late to study them, as their genomes (DNA) for instance are often fragmented, duplicated, and are frankly messed up. Of the cells that can be taken directly from the body for study, fibroblasts grow most readily and have relatively normal genomes. We do use these in our studies and have based much of our work to date on them. However, due to this we now have a skewed picture of what defines a senescent cell. The senescence described in vitro (on glass or plastic dishes), mostly in fibroblasts, with a particular stimulus will, most likely, not represent senescence in different cell types / lineages, with different stimuli, across different tissues and at different ages. We therefore want to characterise senescence in different cells of the body in the context of their microenvironment in this application. Cells can be co-cultured, i.e. two cells types grown together, however this can often only be performed with two cells types, e.g. cancer cells and a single immune cell type (which is often itself a cancer cell). Increasingly it is becoming evident that cells of the immune system 'talk' to each other through release of messaging factors (chemokines and cytokines) and so in order to get an accurate picture of what is happening with regards to the clearance of senescent cells by the immune system we ideally need to study living organisms where these processes are fully competent. Cells grown in organoids (b) or

tissue slices (c) are one step closer to modelling living organisms but cannot currently fully recapitulate living organisms.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This will be the third project licence that has been held be the lab. The estimated number of animals we will use is based on our usage for the previous 5 years. We consult with a professional statistician where possible and the numbers of mice entered into experimental cohorts are carefully considered together with them. However, the majority of our estimated usage (~60%) arises from the breeding of genetically altered mice, despite our breeding strategies being optimal (and under constant review). Note that innovations such as the administration of virus to the lungs or hydrodynamic delivery of plasmid DNA to the liver enhances the numbers of mice we can use in experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A professional statistician is employed by our establishment for the purpose of helping to design our experiments so that we use the optimum number of mice (or indeed plates of cultured cells). As well as consulting with them we also use online tools such as the NC3R's Experimental Design Assistant.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The majority of our mouse usage comes from the breeding of genetically modified lines where unfortunately a large number of surplus animals are generated due to the nature of Mendelian genetic inheritance. We have optimised our breeding strategy to minimise the generation of surplus animals.

We have also adopted techniques which reduce or remove the need to breed mice altogether. Our intravenous injection of plasmid DNA technique for introducing that DNA into the liver (which we refer to as 'hydrodynamic transfection') or our viral administration of DNA into the lungs can produce genetically altered mice without the need for breeding genetically altered animals and thus reduces mouse usage.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This programme of work is exclusively concerned with the use of mice as a model system. Mice have been used in the study of cancer for much of the last century and indeed mouse models of cancer are now really quite refined. Mouse genetics has developed to the point where a researcher can induce cancer simply by adding a completely innocuous substance to their diet or drinking water, thereby inducing the genetic alterations within the genome of those mice.

Our work is concerned with studying the development of cancer within the first few days to weeks after its initiation. As such the majority of our mice will not develop tumours nor the pain and suffering associated with that.

Why can’t you use animals that are less sentient?

Although there are childhood forms of cancer, which our colleagues work on, cancer is primarily a disease of adult animals, and indeed its incidence increases with age, so to use animals at an immature life stage is not appropriate to our studies.

Whereas, arguably less sentient animals can develop cancer, there is always the question as to how well this recapitulates human cancer. Mice offer the possibility to study cancer in a mammalian organism that recapitulates human cancer very well, whereas at the same time they are quick to breed and relatively easy to house.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Using as an example one of the main techniques that we use, hydrodynamic tail vein injection (HDTV). We have extensive experience with using this technique over the last 8 years. We have refined the technique in order to maximise success and minimise adverse effects for the mice. For instance, we have determined an optimum age range for the mice to be injected mice to be between 6 and 9 weeks of age, before they have put on excess weight necessitating increased injected volume. We will attempt continued refinement of the technique going forward during this proposal. There is interest in the potential use of this technique under general anaesthesia however, there is no data demonstrating non-inferiority of successful delivery of the injection to the animal, or the plasmid DNA to the liver, when using this approach. Therefore, during this project we will trial the HDTVi

delivery of our plasmid DNA in awake and anaesthetised animals to analyse the scientific validity of HDTVi under anaesthesia in these key metrics.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The scientific literature includes journals dedicated to the publication of protocols, which often represent best practice and include tips for refinements to techniques. We also are in close communication with other labs that perform the same techniques as us, including discussing any refinements they have made and/or are currently making.

Unless otherwise specified, the work in this project will be undertaken in accordance with the principles set out in the Guidelines for the Welfare and Use of Animals in Cancer Research: British Journal of Cancer (2010) 102:1555-1577 (referred to subsequently as the ‘NCRI Guidelines’) and in the LASA Guiding Principles for Preparing for and Undertaking Aseptic surgery (2010).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The 3Rs of scientific animal work are replacement, reduction and refinement. Refinement is concerned with methods that alleviate or minimize potential pain and distress, thus emphasizing animal well- being. In the UK a national centre for the 3Rs (NC3Rs) was setup in 2004 and it produces a monthly news letter to which we subscribe.

We are also in contact with other groups which are involved in similar research to ours and use similar techniques. This kind of 'word of mouth' is often the best way or staying up-to-date with the latest up- and-coming techniques and advances. There is also a monthly meeting of people using our animal facility, attended by representatives from each lab, where our facility's NACWOs and the NVS provide us with suggestions on the latest refinements and 3Rs advancements.

41. Mitochondrial Regulation of Neuronal Presynaptic Function in Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Neurodegeneration, Synapses, Mitochondria

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project aims to further our understanding of how mitochondria regulate neurons in three areas:

1) Studying the precise location of mitochondria at neuronal synapses, to establish how this regulates synaptic transmission and how this changes in Parkinson's disease,

2) Studying how mitochondria interact with other components of neurons at synapses, such as the endoplasmic reticulum (which also regulates signals that can alter neurotransmission), and how this is altered in Parkinson's disease,

3) Studying whether mitochondria located at synapses are different to mitochondria located elsewhere in neurons.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

A pivotal contribution to the success of complex life has been the evolution of the nervous system, since it enables organisms to respond in a rapid and coordinated manner to their environment.

However, it comes at a high price: the human brain is just 2% of the body's mass, yet demands 20% of the body's energy. Much of this energy is used to power synapses, where neurons connect and communicate with each other. An individual neuron contains many hundreds of synapses, which vary in their activity over time. So neurons need a way to meet changing energy demands from one part of the cell to another, and also from one point in time to another. Mitochondria are perfectly suited to help with these changing demands. Mitochondria are key components of cells that can generate useable cellular energy from fuel sources. Furthermore they are mobile, so they can move around within cells to provide energy to where it is most needed. It follows that mitochondria are frequently located at synapses.

As well as providing energy to support activity at synapses, our recent work has shown that mitochondria can also reduce activity at individual synapses by mopping up a signal that triggers neurotransmitter release at synapses. Furthermore, the ability of mitochondria to move to and from synapses, and to alter the levels of this signal, is impaired in mouse models of Parkinson's disease, suggesting that the balance of energy provision and signal regulation provided by mitochondria is important in maintaining normal synaptic function. Therefore we need a more complete understanding of why mitochondria have this dual role in regulating neuronal synapses, and the molecular mechanisms by which this regulation occurs, and also how this regulation changes in neurological and psychiatric disease. Understanding this form of synaptic regulation could provide new approaches to treating conditions such as Parkinson's disease.

What outputs do you think you will see at the end of this project?

Anticipated outputs include the generation of new scientific knowledge, specifically furthering our understanding of synaptic regulation by mitochondria. The primary output would be peer-reviewed scientific papers, and presentations at scientific conferences.

Who or what will benefit from these outputs, and how?

Impairment of synapses contributes to many diseases, including autism spectrum disorder, schizophrenia, epilepsy and stroke, as well as neurodegenerative disorders (which arise due to the premature death of certain types of neurons, seen in Parkinson's disease, Alzheimer's disease and motor neuron disease amongst others).

In the short term, this program of work will provide insight into synaptic regulation which will benefit research groups working on related areas. In the longer term, given that we know relatively little about how mitochondria support and regulate synapses, this program of work has the potential to be relevant to a wide range of diseases that currently have few if any treatments available. The ultimate goal of this research is to provide additional mechanistic insight into how mitochondria contribute to synaptic dysfunction in neurological and psychiatric disease, and therefore provide novel therapeutic opportunities to treat these diseases.

How will you look to maximise the outputs of this work?

The results from this project will be initially communicated at scientific conferences and via publicly available pre-prints, and then in peer reviewed journals. The nature of the work is highly collaborative and will allow the building of new research teams to continue active investigation.

Species and numbers of animals expected to be used

- Mice: 15000
- Rats: 250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice and rats are used as the lowest vertebrates with a nervous system that closely resembles that of humans. The majority of experiments will be performed on nervous tissue obtained from embryos (wildtype or transgenic mice and wildtype rats) or neonatal mice, with some experiments performed on adult mice. Although some mice with gene mutations that cause Parkinson's disease can develop motor symptoms akin to

Parkinson's, the number of mice so affected will be kept to the absolute minimum, and mice will be sacrificed at the end of the experiments.

Typically, what will be done to an animal used in your project?

We estimate that for the majority of experiments (approximately 75%), mice or rats will be mated, and the pregnant dams sacrificed. Nervous tissue will be obtained from embryos, and grown in culture, then used in subsequent experiments. Neuronal cultures may also be generated from early postnatal mice that have been humanely killed.

In a further 15% of experiments, brain tissue (slice cultures) will be obtained from early postnatal mice that have been humanely killed. Some brain tissue (slices) may be obtained from adult mice that have been humanely killed.

In approximately 5% of experiments, mice will be aged beyond 12 months. These animals may undergo non-invasive behavioural testing that would cause no harm or distress. Brain tissue (slices) may be obtained from these aged animals that have been humanely killed.

In approximately 5% of experiments, mice will undergo surgical procedures under deep anaesthesia to provide access to the brain, during which devices for recording activity of nerve cells or delivery of substances into the brain. Some of these mice will be fitted with cranial windows and head fixation devices to enable microscopy. For all of these experiments, all animals will receive pain relief and will be closely monitored during recovery. In most cases, mice will undergo one or two surgical procedures, with sufficient time between surgeries to allow for complete recovery. In some experiments the mice may have head movements restricted (via head fixation) to allow recording of neural activity and/or reliable presentation of sensory stimuli. In these cases, mice will be habituated to head fixation and the experimental setup to minimise stress and discomfort.

What are the expected impacts and/or adverse effects for the animals during your project?

All rats and the majority of mice will undergo either subthreshold or mild experiences, typically associated with breeding or earclipping for genotyping or identification, and are brief. The vast majority of adult mice carrying mutations that cause Parkinson's disease will be sacrificed before they develop symptoms. Adult Parkinson's mice that are not sacrificed at this early time point will be carefully monitored for development of signs of distress, and sacrificed if this occurs.

For mice undergoing surgeries required for access to the brain, pain resulting from surgical procedures may reach moderate severity for short periods of time immediately following surgery. Animals will be closely monitored for signs of pain after surgery and appropriate analgesia will be provided. Head fixation is expected to result in only mild stress during initial habituation to the experimental apparatus.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All rats and the vast majority of mice (95%) will experience subthreshold severity. Otherwise severities are mild or moderate (5%).

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The proposed work aims to advance our understanding of how mitochondria behave in neurons in a system resembling the intact brain as closely as possible. For this reason, experiments are carried out on neurons grown in culture from mice or rats (75% of experiments), or on brain slices obtained from mice (20%), where normal connections between neurons are reasonably well preserved, or in vivo (5%). Mice and rats have been chosen since these are the lowest vertebrates with a nervous system sufficiently similar to humans to enable conclusions to be drawn that are relevant to the advancement of clinical care.

Which non-animal alternatives did you consider for use in this project?

Wherever possible, alternatives to animals will be used, for example induced pluripotent stem cell- derived neurons from Parkinson's patients, which enables us to examine the effects of Parkinson's- causing mutations in the specific human cells that are affected by disease. We have also searched existing databases of results which we can apply to our research questions, without needing to repeat the experiments in animals ourselves.

Why were they not suitable?

Stem cell-derived neurons do not fully recapitulate synaptic connections in vitro, and so whilst they are a valuable additional model, they are not sufficient to fully replace rodent studies for the planned work.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimate of mouse and rat numbers is based on experimental experience, aiming to balance reduction in use with producing robust, reproducible, and meaningful results. Statistical techniques have been used to estimate the number of mice or rats included in each experimental group, to reduce the risk of false positive or negative results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used the NC3R's Experimental Design Assistant, and employed statistical calculations using analysis software.

Additionally, we use rats as an alternative to mice where non genetically altered neurons in culture or slices are to be studied. Rat neuronal cultures are more robust and easier to maintain than mice cultures, and more neuronal cultures can be generated per animal. Therefore this reduces the overall number of animals that are needed for experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

A key element of the usage of mice is the breeding of genetically-altered animals, therefore we will optimise breeding strategies to reduce surplus animals which would otherwise need to be culled.

The experimental utility of each mouse or rat will be optimised by maximising the number of measurements taken from each animal, and tissue will be shared when possible to reduce the need for mouse or rat use by other groups. The project will use the least number of mice or rats necessary for appropriate statistical power and reproducibility. In all cases, steps will be taken to reduce systematic bias, including blinding of genotype, using both genders where applicable, and appropriately replicating results in independent experiments. For large datasets, expert statistical advice will be sought where required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The experimental methods and models we will employ are all well established as techniques which provide robust and reproducible results carefully balanced against causing unnecessary harm. We use rats to generate neuronal cultures which can then be labelled for proteins of interest and used in experiments. We use mice which carry genetic instructions to tag mitochondria with a fluorescent protein that enables us to visualise mitochondria under the microscope. We also use mice that have genetic instructions that direct this tag to be expressed only in midbrain dopaminergic neurons, which are the neurons most susceptible in Parkinson's, so that mitochondrial function can be studied specifically in these cells. These genetic instructions are harmless to the mice.

We also use mice that carry mutations that cause Parkinson's disease, or that affect mitochondrial function or mobility, which can lead to neurodegeneration, however symptoms only develop after a period of time. This enables us to closely monitor the mice, and humanely sacrifice any that develop distressing symptoms, to minimise harm.

For limited experiments, surgical procedures will be conducted under deep anaesthesia following aseptic technique and mice will be carefully monitored following surgery to ensure complete and uneventful recovery.

Why can’t you use animals that are less sentient?

Rodents are the least sentient animals that have a nervous system similar enough to humans to be able to begin to generalise results, and in which human neurological disease can be modelled. The availability of disease models allows us to test whether new treatments might be effective in humans, and whether side effects are likely.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Refinement relevant to this project would be ensuring that harm to animals carrying mutations that cause neurodegeneration are not kept alive longer than is absolutely necessary for the proposed experiments.

Appropriate analgesia will be used during surgical procedures and mice will be closely monitored during recovery. In experiments involving head-fixation, mice will be gradually habituated to the experimental setup to minimise stress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the ARRIVE, PREPARE, and FELASA guidelines, and we will follow the guidelines set out in LASA Guiding Principles on Preparing for and Undertaking Aseptic Surgery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We closely follow the neuroscience literature relevant to the project, continuously looking for techniques which will allow us to refine the animal work which we conduct. We are also following NC3Rs initiatives closely, both on their website and as disseminated through the institute and university.

42. Investigating Mechanisms of Biomechanical Stress and Inherited Heart Disease (Cardiomyopathy)

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Inherited heart disease, Modelling diseases, Therapeutic approaches to disease, Biomechanical stress

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to better understand a group of human inherited heart diseases called cardiomyopathies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Inherited heart diseases called cardiomyopathies contribute substantially to the burden of heart disease in the UK (Overall cost of heart diseases to the UK economy is estimated to be £19 billion each year, with over 7.6 million people estimated to be living with heart diseases in the UK).

The majority of cardiomyopathies are caused by genetic 'spelling mistakes' in proteins important for cardiac contraction and there is a reasonably good understanding of how they cause disease. This project will focus on less investigated novel group of proteins associated with the hearts ability to sense stress, or other proteins found to be associated with the disease. Alterations in these proteins can lead to detrimental alterations in the hearts structure i.e the heart becomes bigger and stiffer, it may also be unable to work efficiently to sustain a healthy delivery of blood to the rest of the body's organs.

Our work will provide novel understanding of "what goes wrong" at molecular level in the presence of the "spelling mistakes" and we will focus these investigations on signalling networks, e.g. on how the heart senses and responds to mechanical demand (a process called bio-mechanical stress signalling). This will advance our knowledge of which signalling pathways are crucial for maintaining normal cardiac function and how they are affected in the presence of disease-causing "spelling mistakes", both in "traditional" disease genes affecting cardiac contractile units (responsible for generating force) and in newly identified "unconventional" disease genes, e.g. coding for proteins sensing and responding to mechanical demand. The work will help to uncover novel key players in these signalling networks and thereby identify potential therapeutic targets for specific treatments of cardiomyopathies.

What outputs do you think you will see at the end of this project?

This research will help to gain insights into how variations in key proteins responsible for sensing and responding to mechanical demand can cause heart diseases, such as cardiomyopathy. The project will generate mouse models of human disease, by taking "spelling mistakes" identified in patients and introducing them in mice. Our experiments will tell us which cellular pathways 'go wrong' resulting in disease presentation.

Based on the findings and better understanding of the disease-mechanisms, potential therapies can then be developed to treat or prevent these diseases.

Our research will be disseminated to other researchers (e.g. through conferences, peer-reviewed journal articles).

Who or what will benefit from these outputs, and how?

Short term- A deeper understanding about specific gene alterations and bio-mechanical stress signalling pathways linked to cardiomyopathy. Our research and knowledge gained

from this project will be disseminated to other researchers/clinicians e.g. through conferences and peer-reviewed journal articles.

Medium term- The mechanisms and pathways identified will enable the development of therapeutic targets to potentially treat/prevent disease.

Long term- Help to inform Geneticists in the NHS how to interpret certain findings of genetic testing in human patients and how to advise patients and clinical practitioners on the findings. We will also explore how the disease can be ameliorated or treated.

How will you look to maximise the outputs of this work?

To maximise the outputs from this work, our findings will be presented at conferences and published in peer-reviewed original research articles.

Species and numbers of animals expected to be used

- Mice: 7650

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Cardiomyopathy and other heart diseases are complex and involve a wide range of interactions and pathways across tissues and cells that cannot effectively be studied exclusively in vitro. Whole animal models provide a greater insight into the mechanisms of a disease at organ level, over that of cell models and human studies. Mice are sufficiently similar genetically and in terms of heart structure and function to that of humans, despite differences in size and physiology. Introduction of genetic variants identified in human cardiomyopathy can also be achieved efficiently in mouse models. As the diseases we are studying usually occur later in life, adult mice are the most appropriate life stage for most of our work.

Typically, what will be done to an animal used in your project?

Typically, animals possessing genetic alterations relevant to the human condition of cardiomyopathy will be produced and maintained in order to characterise mechanisms of the disease. Non-invasive assessment of structural and functional changes to the heart will be undertaken by echocardiography (an ultrasound investigation of the heart). In mice this is carried out under general anaesthesia to minimise stress to the animal. We will perform this investigation at multiple time points to determine disease progression in adult mice. Some animals will have non-invasive electrocardiographic (ECG) assessment to identify those at risk of arrhythmias in conscious animals.

Some animals that do not show signs of cardiac disease will be challenged by the administration of drugs to induce bio-mechanical stress. These will be delivered over longer periods of time by an osmotic mini-pump implanted under general anaesthesia. Mice will be monitored daily for their general well-being and weighed on a weekly basis. If animals show signs of distress, they will be humanely killed. The drugs given will mimic the effects of high-impact endurance training on the heart and will help to reveal defects in signalling pathways, which may not be visible otherwise in the mouse model.

In other sets of experiments, we will use our insights into “what goes wrong” in cardiomyopathies to test how treatments, which are predicted to reverse these pathways, may ameliorate disease by injection.

At the end of the studies the animals will be killed and their organs will be used for molecular studies or the heart will be excised under terminal anaesthesia for further Langendorff perfusion or cell isolation for assessment of cardiac and cellular function to maximize the data obtained from each animal.

What are the expected impacts and/or adverse effects for the animals during your project?

From previous experience we assume that the introduction of the genetic variants (“spelling mistakes”) will have little effect on the majority of mice, despite being present for the whole duration of the mouse's life. Mice may not show any signs of cardiac disease or signs are subtle and can only be picked up by specialist tests (such as echocardiography, see above). In some cases, mice may develop symptomatic heart failure similar to humans, identified by deep abdominal breathing and lethargy. In this case animals will be humanely killed.

Some animals will undergo mini-pump implantation under general anaesthesia. This will allow the delivery of mechanical stress inducing drugs over the course of 2-6 weeks in order to exacerbate the impact of a genetic variation on heart structure and function.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild severity- approximately 25%
Moderate severity- approximately 75%
What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We use bioinformatics predictions, biochemical experiments and cellular models to understand the impacts of the genetic “spelling mistakes” on heart cells. While these experiments are informative for certain aspects of disease (e.g. they can show binding to other proteins is affected), mouse models are needed to understand the effects of the genetic “spelling mistakes” on the whole organ level, e.g. how these genetic “spelling mistakes” can cause changes to cardiac size and function as well as electric conduction.

In our in vitro experiments, we use human cardiomyocytes derived from blood or skin biopsies via an exciting novel technology called "induced pluripotent stem cells". These cells are human to reflect best real human heart cells and future work will show which aspects of animal work can be replaced by these cells. Over the course of the project we will review and potentially incorporate alternatives as they become available.

We also pursue approaches to use computational modelling (in silico predictions) to reduce the number of animals used in research. This is particularly useful to predict changes in electrophysiology, which can predispose to arrhythmic events.

Which non-animal alternatives did you consider for use in this project?

We have explored the use of stem cell-derived cardiomyocytes. Work on stem cell-derived cardiomyocytes is a parallel core activity in my lab and we are currently replacing animal work where possible.

Why were they not suitable?

Stem cell-derived cardiomyocytes are immature and do not fully recapitulate the complexity of the heart, including many cell types and physical and neurohormonal local controls. Heart diseases such as cardiomyopathies are complex, involving changes in blood flow, oxygen supply to other tissues and changes in heart structure over time, therefore exploration of this disease requires a multi-factorial approach.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Expected effect size and number of animals have been determined through consultation of the literature, previous experience of techniques proposed or through small pilot experiments when possible. we will seek advice from a statistician where applicable.

Furthermore, breeding strategies are designed to produce the required numbers without having excess of unwanted genotypes (e.g. avoiding crossing heterozygous animals with each other, and using only wildtype pairs and homozygous pairs to generate the required genotype.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When designing the experiments, we performed statistical analysis to ensure we use the minimum number of mice per group that will be informative. Importantly, we also employ a variety of approaches to reduce animal usage, including:

1. Using littermate pairs where possible, to reduce variability of genetic and behavioural background

2. Performing and analysing experiments in a blind fashion where possible to reduce investigator- induced bias

3. Using standardised experimental methods to reduce variability 4.The NC3Rs EDA tool will be used to design experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To maximise the information gained from a single animal, we aim to perform multiple in vivo and in vitro analyses. Cell line work (especially using stem cell derived cardiomyocytes) and in vitro manipulations will be employed where possible to reduce animal usage.

Better reporting of research should result in better science and more effective use of animals in experiments. Therefore, our findings will be reported (using the ARRIVE guidelines) in the scientific literature and at conferences, thereby minimising risk for future unnecessary animal experiments conducted by others.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used for all experiments, as methods are available to introduce genetic “spelling mistakes” easily and mice are similar enough to humans in terms of cardiac physiology, e.g. their hearts have four chambers like the human heart.

The team has long-standing expertise and experience in all experimental procedures; we will constantly refine techniques and apply best practice to all animal work.

Cardiac phenotyping

We make extensive use of non-invasive imaging technologies (especially echocardiography). The use of serial non-invasive measurements e.g. echocardiography examinations, reduces numbers since it is not necessary to kill animals at all time points. The majority of animals will undergo at least one echocardiography examination under general anaesthesia with recovery in their lifetime. We will be careful to balance the refinement of imaging at multiple time points with the impact of multiple anaesthesia events. Animals may also undergo electrocardiogram without anaesthesia (tunnel used for restraint due to the short nature of the protocol (<5 minutes)) if arrhythmic event is suspected in line with the genetic alteration they possess.

The aim is to find the earliest time point where impairment of cardiac function can be measured reliably; this is often long before the onset of external clinical signs. We will then use these early "sub- clinical" time points in subsequent experiments e.g. to investigate the hierarchy of specific signalling pathways involved in cardiomyopathy and to study potential interventions.

Drug induced bio-mechanical stress and cardiomyopathy

Drug-induced models of cardiomyopathy will be used as a refinement to induce cardiomyopathy phenotypes in genetically altered mice (and wild-type control mice of the same genetic background for comparison) where scientifically valid instead of applying the more invasive aortic banding procedure, used elsewhere. While aortic banding is a commonly used approach to induce pressure overload cardiomyopathy, it is invasive and requires thoracotomy (“open heart surgery”). Drug-induced models of cardiomyopathy are relatively less invasive, and have intrinsically lower inter-animal variation and require less time than aortic banding to produce a phenotype. We will aim to deliver drugs via implanted osmotic mini-pumps wherever possible, especially for prolonged treatment times to minimise the need for frequent injections. We will adhere to the maximum volumes and frequencies given below (applies to all protocols and is based on LASA guidelines).

Investigation of therapeutic approaches to cardiomyopathy

For one of our models of cardiomyopathy, we have shown that a variation in the DNA causes the lack of a specific protein, important for heart structure. Based on this, we are now investigating whether expressing more of the protein in the heart can ameliorate disease. For this we introduce the missing protein into the heart with the help of an adeno-associated virus (AAV). Pilot experiments carried out in wild-type animals (C57bl6) will be used to establish optimal dosing for therapeutic treatments through injection of these novel reagents. This will be given via intravenous injection adhering to the maximum volumes and frequencies outlined in the LASA guidelines We aim to establish the lowest dose to give a robust read-out, but with minimal adverse effects. Treatment will then be given to a genetically altered mouse model for cardiomyopathy to observe the efficiency of the treatment to alleviate disease.

Why can’t you use animals that are less sentient?

Zebrafish hearts only have two chambers and therefore cannot recapitulate function and physiology of the human heart. The mouse is the least sentient mammal that is most similar to humans in terms the way their heart works (e.g. four chambered morphology) and specific disease pathways (e.g. how it senses and responds mechanical demand). Therefore, we can avoid working with larger mammals such as dogs or pigs.

As the disease we investigate are adult onset in humans, it requires us to work with adult mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Substances that require multiple dosing regimens will be delivered via osmotic mini-pumps which require only minimal surgery to be implanted. This slow release device means we can avoid twice-daily injections of the animals, hence reducing stress and discomfort in the mice.

Subcuticular sutures rather than wound clips will be used where possible, for wound closure following mini-pump implant. Sutures are flexible and therefore, are more comfortable for the animal.

Analgesic will also be given before animal undergoes surgery for implant and after when needed.

We will utilise refined handling techniques to reduce the distress that animals experience during handling.

For non-invasive electrocardiogram measurements, animals will be placed into a secured tunnel to remove the need for anaesthesia and to minimise stress to the animal. While for non-invasive echocardiography, light anaesthesia will be used for restraint to reduce stress of the animal during the procedure.

For all procedures, the mice will be carefully monitored and if adverse events are observed, monitoring will increase in frequency and steps will be taken to alleviate them or the affected mice will be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the PREPARE guidelines to ensure our experiments are planned and conducted in the most refined way. LASA guidelines will be consulted to ensure correct dose volumes are selected for

the specific route of injection/blood sampling. We will also stay up to date with the NC3R website resources and guidance on the best and most refined practices. All the data will be published according to the ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly monitor the guidance given by the NC3R's website as well as emails circulated by the establishments 3R’s committee, making use of the online resources to ensure the project is carried out efficiently and using the best methods for animal welfare.

43. Pathophysiology of Vascular and Renal Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

kidney, vessels, diabetes, hypertension

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our research programme aims at understanding the mechanisms of vessel and kidney disease in animal models with the goal of finding new treatments that could subsequently be applied to humans.

Specifically, our work will focus on novel mechanisms involved in organ repair in diseases. We plan to alter the levels of genes, proteins (factors), in animals and subsequently study their role in normal and disease (diabetes, hypertension) conditions. If these factors demonstrate a protective role in treating a disease of the vasculature or kidney, future

work will aim at developing new medicines that could be utilised to treat/cure diseases in humans.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Vascular and renal diseases are serious illnesses and, at times, life threatening. A suboptimal vascular/renal function (closely linked together) leads to illness, and ultimately death. Although people can be treated with drugs that help vessels/renal function, the prevention of these devastating diseases is crucial for the well-being of patients. Diabetes and hypertension are the major causes of vascular/renal disease. Diabetes and hypertension affect approximately 600 million people worldwide. High blood glucose and hypertension drive diseases that lead to vessels/renal damage. The understanding of vessels/renal diseases and their causes (e.g.: diabetes, hypertension), will help health care professionals in finding new therapeutic approaches for the prevention of vascular and kidney disease.

Our studies aim at understanding the mechanisms of vessels/renal disease, which will help the assessment of new therapies, which hopefully will be of benefit to patients.

What outputs do you think you will see at the end of this project?

This work will provide novel information on the molecular processes underlying the regulation of vessel and renal function, and on the specific role of the kidney in the regulation of water, small solutes/molecules, in physiology and in disease conditions.

Specifically, this work will contribute towards the identification and understanding of novel pathways believed to contribute to vessels, kidney glomerular capillaries and renal tubular cells dysfunction in diseases. The understanding of these pathways will have a significant impact on the identification of potential novel targets for treatment in the field of vascular medicine and nephrology. The growing epidemic of diabetes and hypertension and its associated vessels and kidney disease call for new treatments to be able to maximize end-organ protection thereby preventing or delaying progression of vessels and/or renal injury.

Who or what will benefit from these outputs, and how?

This work will help identifying novel pathophysiological mechanisms of vessels/renal disease (short term benefits - publications and dissemination to international scientific society) and potential novel target for treatment in animals and humans (long term benefits - translation).

How will you look to maximise the outputs of this work?

This works has been planned with the collaboration of different institutions in the UK and abroad.

Results will be presented at scientific meeting and to the lay community and published in scientific Journals.

Dissemination of information will include positive and negative findings as well as methodological approaches and refinements which may be useful to other researchers.

Species and numbers of animals expected to be used

- Mice: 3500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our experimental plan uses the least sentient species (e.g.: mice) which we can use to model diseases and test our proposed treatment strategies.

We will study genetically modified (and wild-type) adult mice to define the effect of diabetes, administration of specific diets (e.g.: high salt, high fat) and infusion of angiotensin-2 on vascular and renal function/blood pressure.

Further we will modify specific cellular pathways to generate a proof of principle for potential new treatments that could, in the future, be translated to humans.

Typically, what will be done to an animal used in your project?

In this project mice may:

1) become diabetic and/or develop high blood pressure (hypertension). Diabetes will be induced by either the injection of a substance that inactivates the insulin production in the pancreas, or by diets rich in fat (or combination of the two), or by using mice that have a genetic alteration or which makes them susceptible to developing diabetes naturally. Both are well defined and characterised animal models of diabetes. The housing and care of animals will be adjusted when animals become diabetic, for example by providing extra water and bedding to keep them comfortable.

2) become hypertensive by administration of agents or diets (with different salt content) that cause a raise in blood pressure. Animals will be monitored for progression of disease. This will involve taking small blood and urine samples. Some animals might undergo surgery to implant devices for the delivery of molecules and/or measurement of

blood pressure. After surgery animals will receive analgesia and will be monitored closely during their recovery.

In our experiments, we will study specifically the vessels and the kidney using non-invasive techniques which will allow us to study their function in health and disease. The presence of diabetes should only cause minimal impairment of vessels/ renal function. Animals may be kept in metabolic cages, and we do not expect any significant impediment from repeated experimental procedures.

3) At the end of the study animals will be humanely killed and tissues collected for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

For most animals used, no adverse effects are expected either in wild-type or genetically altered animals.

Although not anticipated, animals will be monitored regularly for signs of distress or suffering such as weight loss, abnormal behaviors such as lack of grooming, hunching or reduced mobility.

Diabetes may induce a degree of weight loss that is minimised by the specific model utilised to induce diabetes and/or by administration of insulin. The length of diabetes will be for 20 weeks maximum (usually diabetes occurs after 10-14 days from the first administration of streptozotocin).

Mice may be implanted with telemetry devices for blood pressure determination and mini-pump for angiotensin infusion. Mice will be closely monitored after surgery.

Individually caged animals will be adapted in metabolic cages by using home cage bedding for few days. Urine collection will be performed during the last 24 hours, home cage bedding will be removed during this time. Maximum continuous time in metabolic cages will be up to 5 days.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

This project is expected to use 3500 animals.

~60% animals will experience a mild severity banding (P1)

~40% animals will experience a moderate severity banding (P2, P3 and P4)

In case of sudden health deterioration, animal will be treated or humanely killed.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The physiology and pathophysiology of the vessels/kidney system is regulated by a myriad of factors that function as links between different cells, tissues, and organs. To study organ physiology, we need to conduct experiments in mammals, such as mice. This approach maximises the potential to translate our discoveries to humans.

In our clinical practice, we study human patients with vessels and renal disease, and we are limited to the nature of studies we can perform; patients will often have been receiving treatments for some years and it is rarely possible to study patients before they develop disease or in the early phase of the disease itself. As a result, we need to use animal models of disease that allow us to study the mechanisms of diseases from the earliest stage and during disease progression; this will allow us to monitor the effect of specific molecules, in a controlled and standardised setting.

Further, the study of different organs and their interaction is only possible in animals. By using animals, we can investigate the contribution of specific pathways in a specific tissue in different experimental conditions.

Our plan of work clearly reflects the need to study the whole animal. Where possible we will utilise non- animal laboratory-based approaches to complement the in vivo work.

Which non-animal alternatives did you consider for use in this project?

Where possible, the use of cells grown in the laboratory (e.g.: human glomerular endothelial cells, human mesangial cells, mouse tubular cells) or organoids (to replace animal work) will be utilised to answer our questions.

We will continue to use in vitro methods/techniques to validate the efficacy of novel potential intervention compounds or strategies before considering animal studies.

The in vitro cell/organoid work will also complement the in vivo work by answering questions that cannot be answered in the whole organisms such as changes in the activation of specific molecules and cellular signaling pathways.

Why were they not suitable?

Use of cells or organoids is a close approximation of animal (whole organism) physiology and pathology, but it cannot adequately answer some of the questions we want to ask in vivo.

The complex interplay between different organs, tissues and cell types seen in vascular and kidney physiology/diseases cannot be modeled sufficiently using cells/organoids or other non-animal models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals is based on breeding strategies and experimental plan programmed in the next 5 years of research.

We have been generating preliminary data. Animal estimates are based on our vast experience in breeding and using animals in the experiments proposed. Our previous experiments have been peer and statistician reviewed during the publication process.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We established methods to reduce the number of animals studied and enhanced animal well-being. As an example, after terminal anaesthesia we can isolate one of the two kidneys after clamping the renal artery/vein before the animal is perfused with fixative. This allows us to obtain tissue (kidney) from the same animal that is suitable for different experimental procedures/analysis allowing a reduction in animals to be studied.

Good planning of experiments and regular interaction with colleagues and statisticians significantly helps towards a reduction in the number of animals utilised in our research.

We have setup and will utilise experimental techniques that maximise the acquisition of experimental information (e.g.: physiological parameters analysis such as blood pressure measurements, paralleled by blood, urine, tissue collection for different determinations) from the same animal without significant increase in harm, thereby reducing the animals required.

Breeding strategies have been planned to maximise the number of genetically altered animals and controls, whilst minimising the numbers of animals used.

Our previous experience, preliminary data, interaction with other laboratories, and previous literature will help us with the design and statistical power of our experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Genotyping will be planned pre-weaning. By avoiding inefficient or delayed genotyping we can avoid producing a surplus of animals.

We have previous experience in breeding genetically modified animals, and we have established efficient breeding strategies to reduce the number of animals bred throughout this license.

Some preliminary and pilot work has been carried out and this would therefore help in reducing the animal numbers further. Pilot studies allow accurate power calculations and help plan the adequate number of animals required in the experiments.

We will be using similar mice strains utilised in the past; our knowledge and experience will allow more efficient experiments.

For blood pressure determination, the use of telemetry will provide a more detailed information on the blood pressure day profile and better-quality measurements that could help reduce the number of animals needed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Experimental models of diabetes include genetic models, diet induced models, and models which use drug-mediated damage of the insulin producing cells.

We opted for the induction of diabetes by injection of a substance called streptozotocin. Streptozotocin will be administered at a low-dose for five consecutive days. Reports suggest that multiple low-dose injections of streptozotocin induces a delayed but progressively increasing state of hyperglycaemia in mice. This method does not

completely damage the insulin producing cells of the pancreas and usually does not warrant insulin administration (injections), nor lead to dramatic weight loss. The slow progression of diabetes in this model (which allows the maintenance of a "diabetes status" for 26-30 weeks) allows investigators to investigate the long-term effects of diabetes on tissues and organs (e.g.: time course for blood pressure, albuminuria) which will allow better and solid information to aid answering the scientific questions posed.

We may administer streptozotocin alone or in conjunction with high fat diet. Streptozotocin administration alone (causing reduced insulin secretion) and streptozotocin administration + high fat diet (causing reduced insulin secretion and insulin resistance) are two well-established models of diabetes validated for the study of diabetic kidney disease.

The choice of model between streptozotocin administration alone or streptozotocin administration + high fat diet will depend on the pathways that we will study as per reports in the literature.

For blood pressure measurements, we will use telemetry (involving surgery) and tail cuff methodology. As model of hypertension, we may utilise angiotensin-2 administration (subcutaneously with mini- pump), or high salt diet up to 4% sodium. These interventions are established models that result in a consistent increase in BP around 10-30 mmHg.

Blood pressure telemetry methodology offers state-of-the-art blood pressure determination with decreased need for multiple repeated measures, despite the need of a surgical procedure.

To ensure that animals do not suffer unnecessarily because of repeated testing (e.g.: assessment of blood pressure, glucose control, metabolic cages for water/salt balance), we will only conduct the minimum number of procedures needed to answer our scientific research questions and will ensure that animals have a suitable recovery period in-between procedure.

Why can’t you use animals that are less sentient?

We will study mice as these are least sentient animal that can be used to mimic human disease conditions and can be used to investigate specific genetic manipulations of relevance to vessel and renal physiology and pathophysiology of diseases.

Mice are mammals and will help the translatability of our observations to humans. We therefore need complex organisms where we can study different tissues/organs and their role in physiology and disease settings.

We will implement terminal anaesthesia for tissues and blood collection at the end of the study so the animal is unaware of the procedure as is under anaesthesia throughout and is not recovered.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In diabetic mice, where necessary, we might administer insulin to prevent excessive weight loss.

Use of low doses of streptozotocin with dietary changes (high fat diet) to produce insulin deficiency in conjunction with insulin resistance may be utilised to monitor specific pathways/endpoint that are more pronounced with this experimental approach.

In post-surgery (telemetry), we will utilise analgesia for pain relief, and we will continue to improve and refine the post operative care.

Refinement of our techniques with animal work is always ongoing. We always try to improve our animal research techniques (e.g.: such as bedding in metabolic cages for adaptation to long periods) by

animal monitoring especially as disease progresses (e.g.: long diabetes duration), by improvements of our surgical skills and reassessment of post-operative care routines and adjustments where necessary to optimise management of pain.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All animal work will be conducted with the aim of being compliant with the ARRIVE guidelines for publication of animal research. This ensures minimum standards are met with regards to experimental design, powering, randomisation, inclusion/ exclusion criteria, blinding, outcome measure reporting, and statistical methods.

These guidelines also set out the minimum standards for reporting experimental animal use and experimental procedures, including the rational for proposed procedures to ensure experiments are performed with sufficient scientific rigor without compromised animal welfare.

Further we will regularly consult the NC3Rs website.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will be having continuous discussion with colleagues and staff (e.g.: veterinary, the Named Animal Care and Welfare officer and colleagues) within our animal facility.

We are subscribed to the NC3Rs newsletter.

44. Understanding the Role of the Non-Coding Genome in Brain Development, Function and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Gene Therapy, Cell Therapy, Advanced therapeutics, ATMPs, Lung gene transfer

Animal types	Life stages
Mice	neonate, juvenile, pregnant, adult, embryo, aged
Rats	juvenile, neonate, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to develop advanced therapeutics, namely gene and cell therapy-based treatments for pulmonary and non-pulmonary diseases by using topical delivery of gene and cell therapy products to the lungs.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Many diseases such as cystic fibrosis, pulmonary alveolar proteinosis, alpha-1 antitrypsin deficiency, interstitial pulmonary fibrosis and thrombotic thrombocytopenic purpura have significant unmet clinical needs (see details below). Gene and cell therapies have been successfully developed for several diseases indications and offer promise for others.

Cystic fibrosis (CF)

CF is a genetic disease (most prominent in Caucasians) with an incidence of approximately 1:2000 live births and approximately 100,000 people affected in Europe and North America alone. Although life expectancy has significantly increased from 0.5 yrs in the 1940s to approximately 35 years to date, treatment options are expensive, time-consuming and not sufficient to prevent pre-mature mortality in a large number of patients.

Pulmonary alveolar proteinosis (PAP)

PAP is a rare genetic lung disease (0.1 to 1 cases per million) characterised by the presence of lipids in the lungs, leading to decreased gas exchange and defective host defence. A molecule called GM- CSF is critical for the reduction of lipid deposition in the lungs. In some forms of the disease, patients develop antibodies to GM-CSF which reduce its effectiveness and cause lipid deposition.

Alpha 1 antitrypsin deficiency (AATD)

A1AD is one of the most common genetic diseases. Worldwide, an estimated 1.1 million people have A1AT deficiency Individuals with A1AD may develop chronic obstructive pulmonary disease (emphysema) during their thirties or forties even without a history of smoking, though smoking greatly increases the risk. Symptoms may include shortness of breath (on exertion and later at rest), wheezing, and sputum production. Symptoms may resemble recurrent respiratory infections or asthma. Conditions associated with alpha-1 antitrypsin deficiency, occurring due to paucity of AAT in circulation allowing uninhibited inflammation in lungs

Thrombotic thrombocytopenic purpura (TTP)

TTP is a rare (~1 case per 200,000) but life-threatening disease which is characterised by the presence of blood clots (thrombi) within the vasculature, commonly within the brain, heart and kidney. In the majority of cases TTP is caused by the production of antibodies against a protein called ADAMTS13. ADAMTS13 is protein which is involved in reducing inappropriate formation of blood clots (thrombi) in small blood vessels. If left untreated, these thrombi can cause organ failure and death in the majority of cases.

Interstitial pulmonary fibrosis (IPF)

In 2012, about 32,500 people had IPF in the UK and around 50 people in every 100,000 had been diagnosed at some time in their life with IPF. The cause of IPF is unknown but certain environmental factors and exposures have been shown to increase the risk of getting IPF. Cigarette smoking is the best recognized and most accepted risk factor for IPF, and increases the risk of IPF by about twofold. Other environmental and occupation exposures such as exposure to metal dust, wood dust, coal dust, silica, stone dust, biologic dusts coming from hay dust or mold spores or other agricultural products, and occupations related to farming/livestock have also been shown to increase the risk for IPF.

Current treatments include daily physiotherapy, pancreatic enzyme supplementation, intravenous or inhaled antibiotics, administration of anti-inflammatory drugs and bronchodilators in many cases, but are not sufficient to increase life-expectancy. A number of drugs that potentiate or correct the defect protein have recently been licensed, but their long-term efficacy and safety is not yet proven. In addition, these drugs are ineffective in patients with mutations that completely prevent expression of the mutated protein (~15% of patients).

What are the problems with current treatments which mean that further work is necessary?

PAP

The standard of care for PAP is mechanical removal of the accumulated lipids by "washing out" the lungs with many litres of liquid. In aPAP, daily sub cutaneous as well as twice daily inhaled GM-CSF protein administration has been shown to be well tolerated and effective. GM-CSF is not licenced for PAP and has to be imported on a named-patient basis and used off-licence. Costs vary but are around

£30,000 for 6 months treatment. Gene therapy offers an alternative approach and may overcome some of the problems and limitations encountered by protein replacement therapy.

AATD

Treatment of lung disease may include bronchodilators, inhaled steroids, and, when infections occur, antibiotics. People with lung disease due to A1AD may receive intravenous infusions of alpha-1 antitrypsin, derived from donated human plasma. This augmentation therapy is thought to arrest the course of the disease and halt any further damage to the lungs. Long-term studies of the effectiveness of A1AT replacement therapy are not available.

TTP

Most current treatments involve regular blood transfusions and in some cases removal of antibodies to ADAM13 from blood of patients. Individuals usually develop acquired TTP from 20-60 years of age, with one third of cases becoming chronic. Individuals suffering an

acute TTP attack undergo plasma exchange, which has reduced mortality rates from 90% to 20%. Due to the high treatment burden and reliance on donor plasma, a novel therapy for TTP is required.

IPF

There is currently no cure for IPF. The clinical course can be unpredictable. IPF progression is associated with an estimated median survival time of 2 to 5 years following diagnosis.

What outputs do you think you will see at the end of this project?

We have a strong track record for translational bench-to-bedside research and have carried our several gene therapy trials. At the end of this project, we anticipate to conduct further gene or cell therapy trials for diseases such as cystic fibrosis, alpha 1 antitrypsin deficiency, pulmonary fibrosis and thrombotic thrombocytopenic purpura. Our research will certainly lead to conference presentations and peer reviewed publications. In addition, we anticipate that our research will identify new treatment options for the named diseases which currently have an unmet clinical need.

Who or what will benefit from these outputs, and how?

Our disease targets include cystic fibrosis, pulmonary alveolar proteinosis, alpha-1 antitrypsin deficiency, interstitial pulmonary fibrosis and thrombotic thrombocytopenic purpura. We anticipate, that these patient cohorts will benefit, if our translational research programme is successful. More specifically:

Short-term: Proof-of-concept that gene and cell therapy can improve or stabilise disease in our pre- clinical disease models.

Medium term: Translation of gene and cell therapies into clinical trials.

Long-term: Licensing and adopting of gene and cell therapy-based treatments for disease with unmet clinical needs.

How will you look to maximise the outputs of this work?

Collaboration: Approximately 20 years ago we founded a consortium consisting of scientists and clinicians across several UK Universities. We form the largest group worldwide involved in developing advanced therapeutics for pulmonary diseases.

We disseminate new knowledge through interaction with academics at conferences and publications. Importantly, we publish all results regardless if they are proving or disproving our hypothesis.

In addition we are actively involved in patient and public education around advanced therapeutics.

Species and numbers of animals expected to be used

- Mice: Over 5 years we anticipate to use approximately 5000 mice
- Rats: Over 5 years we anticipate to use approximately 100 rats

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Over 95% of animals used in our studies are normal healthy mice or mice in which disease is induced. The vast majority of mice used will be treated when more than 6 weeks old. We anticipate that advanced therapeutics for the disease indications that we are describing in this license will be administered to adult populations rather than children. Mice > 6 weeks old therefore mimic the intended age of the proposed patient cohorts.

Mice are used because relevant disease models have been generated through our ability modify and shut-down genes.

Occasionally, but very infrequently, we confirm data generated in rats. This is particularly relevant for research related to cystic fibrosis. CF knockout mice do not develop CF lung disease, whereas CF rats develop characteristic pulmonary features of CF.

Typically, what will be done to an animal used in your project?

A typical experiment (see more details below) includes administration of a gene transfer agent or of gene modified cells to the lungs of mice using non-invasive delivery methods via nose or mouth. At specific time-points we may collect small volumes of blood via the tail vein or will collect urine. In some animals we may collect breath under anaesthesia by putting mice into a breath collection chamber or we may image gene expression in anaesthetised mice. Most animals (~90%) are humanely killed within a few months post gene/cell transfer. A small number of animals is followed up for longer periods (max 24 months) to assess duration and impact of the treatments on the disease . In addition to wildtype mice, disease-specific genetically modified mice may be used. In a typical experiment animals will be treated with an advanced therapeutic once and culled at a specific time-point post treatment.

Please see below specific details describing the typical protocol for mice and rats on this license.

Life-history: typical scenario protocol for mice

1. Determination of destruction of specific genes (used to generate a model with lung disease). This will for example include taking an ear punch from the animal.

2. Anaesthesia (not more than 12 times over at least 6 months)

3. Administration of the novel drug to the nose, which is then "inhaled" into the lung (animals may receive 1 to 12 doses over 6 months)

4. Blood and urine sampling (typically 6-10 collections over 6-12 months)

5. Collection of breath under recover anaesthesia and/or lung function measurement under terminal anaesthesia (typically 1 measurement)

6. Humane killing

Proportion of mice/rats treated using the typical protocol: >90% Severity limit of the typical protocol: mild

Some animals (<5%) may be housed and observed for up to 24 months

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority (>95%) of our procedures are classified as sub-threshold or mild and do not induce pain or discomfort. Occasionally animals show signs of being unwell for a few hours after treatment (most often this will be visible by looking at the fur, with raised hair (piloerection) being a common sign. The need for human killing of animals post-treatment due to side-effects is extremely rare.

The impact of a typical experiments described above may include a slight and transient discomfort while collecting an ear punch. Gene transfer to the lung may induce transient local inflammation, which generally resolves within 24 hours after treatment and may result in raised hair. Blood sampling via a tail vein may cause transient discomfort due to needle prick. Collection of breath and imaging protocols will be performed under anaesthesia.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice:

95% mild, 5% moderate

Rats:

100% mild

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We aim to develop advanced therapeutics, namely gene and cell therapy-based treatments for pulmonary and non-pulmonary diseases by using topical delivery of gene and cell therapy products to the lungs. Many diseases such as cystic fibrosis, pulmonary alveolar proteinosis, alpha-1 antitrypsin deficiency, interstitial pulmonary fibrosis and thrombotic thrombocytopenic purpura have significant unmet clinical needs. Gene and cell therapies have been successfully developed for several diseases indications and offer promise for others.

As part of our translational research programme we have to assess efficacy and duration of gene expression, as well as toxicity in relevant rodent disease models. Assessment of transduction efficiency and duration of expression requires all cell types that our vector transduces (eg ciliated airway epithelial cells, goblet cells, Club cells, basal cells and pneumocytes) as well beating cilia to be present. Assessment of toxicity and inflammatory responses requires a model in which inflammatory cells such as neutrophils, lymphocytes and macrophages can migrate from the bloodstream into the lungs in response to a challenge such as vector administration. These conditions are only provided in living animals.

Which non-animal alternatives did you consider for use in this project?

In parallel to work in mice we conduct studies in human air liquid interface cultures, human precision cut lung slices and in ex vivo lung perfusion models.

Why were they not suitable?

However these human ex vivo models do not have an intact immune system which impacts on toxicity and efficacy and also don't allow us to assess changes in relevant disease biomarkers.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The projected number of animals in the license reflects the number necessary to achieve the scientific objectives outlined in the programme of work and covers development of new therapies for a number of diseases with unmet clinical needs. The numbers are also based on our previous experience generated over the last 10 years as part of 2 previous project licenses and estimates of the requirements of translational research studies by us and scientists. Projected n numbers also reflect requirements of the regulators (eg bodies such as the Medicines and Healthcare products Regulatory Agency (MHRA)).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have over 25 years experience designing mouse experiments and always aim to minimise n numbers as much as possible through:

- randomisation

- validation of endpoints and biomarkers in pilot experiments to generate baseline data for subsequent power calculations.

- careful selection of endpoints and biomarkers to reduce variability and hence n numbers

- use of the NC3R EDA tool

- ensure that operators are trained to high standards

- careful planning and discussion of experiments to ensure all appropriate control groups are included in the design

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We routinely assess and adapt the size and output of our breeding colonies.

As mentioned above we conduct pilot studies to generate data for subsequent power calculations.

We carefully archive materials from control animals (eg blood, DNA, other body fluids) and reuse this material during analysis of experiments where appropriate.

We use tissues as efficiently as possible (for example by tying of the bronchus to the left lung for RNA extraction before fixing the right lung for histology)

We frequently use a histology core facility to ensure optimal processing, cutting and staining of tissue sections.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will mainly use wildtype mice and genetically modified murine disease models. AATD, TTP and PAP mouse models are mild, breed normally and do not show outward signs of disease. CF knockout mice frequently suffer from intestinal disease leading to high post-weaning mortality. However, we have in the past and will continue to use refined CF models in which the intestinal disease has been corrected through more sophisticated molecular biology strategies.

Gene transfer is performed through topical non-invasive administration to the mouse lung and sometimes through intravenous injections or nebulisation. These methods are minimally invasive.

In some experiment wildtype rats may be used to confirm studies in a larger rodent model.

Why can’t you use animals that are less sentient?

Mouse models are the most commonly used model for gene transfer studies. Although the lung structure and cell composition is not identical to human lung, mice present the best low ranking species for these studies.

We can't terminally anaesthetise mice because for assessment of efficacy and toxicity after gene and cell therapy, the animal has to be alive for indicated times post-dosing.

Some of our gene transfer agents achieve gene expression for prolonged periods of time (sometimes for the life-time of the animal after a single dose). Long-term follow up of efficacy and toxicity is therefore necessary in some experiments.

On rare occasions we may conduct gene transfer in neonates to mimic treatments of babies/children in which the organs are still growing.

In contrast to CF knockout mice, CF knockout rats develop certain features of CF lung disease. We will use wildtpye rats in some experiments to characterise the expression profiles of our advanced therapeutics in rats to allow future studies in CF knockout rats.

Non-protected animals (e.g. invertebrates such as insects, decapods, nematodes) or less sentient animals (eg zebrafish) cannot be used to meet some or all of your objectives of our studies largely. We require a model with lung structure and cell composition as well as the immune system that closely mimic human.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

If required for some experiments we may increase post-treatment monitoring and post-treatment care.

Our experiments are generally mild and do not require any post-operative pain management. However we will seek advice from experts if pain management is required.

In our experience provision of wet food in the cages enhances post-treatment care following general anaesthesia and we provide wet food.

In regular meetings, we also share experiences in animal handling amongst the team to ensure everybody uses the most advanced methods

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are several best practise guidelines that are published and that we are fully aware off and will adhere to. Some examples are listed here: www.aaalac.org/resources;www.reference- resourcesarchanimaltraining.com; (https://norecopa.no/PREPARE).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will carefully review all information communicated by the home office and establishment staff/relevant named persons as well as our establishments 3Rs group. In addition we will keep updated with the NC3Rs website and relevant other activities.

45. Development of Advanced Therapeutics for Pulmonary and Non-Pulmonary Diseases through Topical Delivery to the Lung

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Gene Therapy, Cell Therapy, Advanced therapeutics, ATMPs, Lung gene transfer

Animal types	Life stages
Mice	neonate, juvenile, pregnant, adult, embryo, aged
Rats	juvenile, neonate, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to develop advanced therapeutics, namely gene and cell therapy-based treatments for pulmonary and non-pulmonary diseases by using topical delivery of gene and cell therapy products to the lungs.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Many diseases such as cystic fibrosis, pulmonary alveolar proteinosis, alpha-1 antitrypsin deficiency, interstitial pulmonary fibrosis and thrombotic thrombocytopenic purpura have significant unmet clinical needs (see details below). Gene and cell therapies have been successfully developed for several diseases indications and offer promise for others.

Cystic fibrosis (CF)

CF is a genetic disease (most prominent in Caucasians) with an incidence of approximately 1:2000 live births and approximately 100,000 people affected in Europe and North America alone. Although life expectancy has significantly increased from 0.5 yrs in the 1940s to approximately 35 years to date, treatment options are expensive, time-consuming and not sufficient to prevent pre-mature mortality in a large number of patients.

Pulmonary alveolar proteinosis (PAP)

PAP is a rare genetic lung disease (0.1 to 1 cases per million) characterised by the presence of lipids in the lungs, leading to decreased gas exchange and defective host defence. A molecule called GM- CSF is critical for the reduction of lipid deposition in the lungs. In some forms of the disease, patients develop antibodies to GM-CSF which reduce its effectiveness and cause lipid deposition.

Alpha 1 antitrypsin deficiency (AATD)

A1AD is one of the most common genetic diseases. Worldwide, an estimated 1.1 million people have A1AT deficiency Individuals with A1AD may develop chronic obstructive pulmonary disease (emphysema) during their thirties or forties even without a history of smoking, though smoking greatly increases the risk. Symptoms may include shortness of breath (on exertion and later at rest), wheezing, and sputum production. Symptoms may resemble recurrent respiratory infections or asthma. Conditions associated with alpha-1 antitrypsin deficiency, occurring due to paucity of AAT in circulation allowing uninhibited inflammation in lungs

Thrombotic thrombocytopenic purpura (TTP)

TTP is a rare (~1 case per 200,000) but life-threatening disease which is characterised by the presence of blood clots (thrombi) within the vasculature, commonly within the brain, heart and kidney. In the majority of cases TTP is caused by the production of antibodies against a protein called ADAMTS13. ADAMTS13 is protein which is involved in reducing inappropriate formation of blood clots (thrombi) in small blood vessels. If left untreated, these thrombi can cause organ failure and death in the majority of cases.

Interstitial pulmonary fibrosis (IPF)

In 2012, about 32,500 people had IPF in the UK and around 50 people in every 100,000 had been diagnosed at some time in their life with IPF. The cause of IPF is unknown but certain environmental factors and exposures have been shown to increase the risk of getting IPF. Cigarette smoking is the best recognized and most accepted risk factor for IPF, and increases the risk of IPF by about twofold. Other environmental and occupation exposures such as exposure to metal dust, wood dust, coal dust, silica, stone dust, biologic dusts coming from hay dust or mold spores or other agricultural products, and occupations related to farming/livestock have also been shown to increase the risk for IPF.

Current treatments include daily physiotherapy, pancreatic enzyme supplementation, intravenous or inhaled antibiotics, administration of anti-inflammatory drugs and bronchodilators in many cases, but are not sufficient to increase life-expectancy. A number of drugs that potentiate or correct the defect protein have recently been licensed, but their long-term efficacy and safety is not yet proven. In addition, these drugs are ineffective in patients with mutations that completely prevent expression of the mutated protein (~15% of patients).

What are the problems with current treatments which mean that further work is necessary?

PAP

The standard of care for PAP is mechanical removal of the accumulated lipids by "washing out" the lungs with many litres of liquid. In aPAP, daily sub cutaneous as well as twice daily inhaled GM-CSF protein administration has been shown to be well tolerated and effective. GM-CSF is not licenced for PAP and has to be imported on a named-patient basis and used off-licence. Costs vary but are around

£30,000 for 6 months treatment. Gene therapy offers an alternative approach and may overcome some of the problems and limitations encountered by protein replacement therapy.

AATD

Treatment of lung disease may include bronchodilators, inhaled steroids, and, when infections occur, antibiotics. People with lung disease due to A1AD may receive intravenous infusions of alpha-1 antitrypsin, derived from donated human plasma. This augmentation therapy is thought to arrest the course of the disease and halt any further damage to the lungs. Long-term studies of the effectiveness of A1AT replacement therapy are not available.

TTP

Most current treatments involve regular blood transfusions and in some cases removal of antibodies to ADAM13 from blood of patients. Individuals usually develop acquired TTP from 20-60 years of age, with one third of cases becoming chronic. Individuals suffering an acute TTP attack undergo plasma exchange, which has reduced mortality rates from 90% to 20%. Due to the high treatment burden and reliance on donor plasma, a novel therapy for TTP is required.

IPF

There is currently no cure for IPF. The clinical course can be unpredictable. IPF progression is associated with an estimated median survival time of 2 to 5 years following diagnosis.

What outputs do you think you will see at the end of this project?

We have a strong track record for translational bench-to-bedside research and have carried our several gene therapy trials. At the end of this project, we anticipate to conduct further gene or cell therapy trials for diseases such as cystic fibrosis, alpha 1 antitrypsin deficiency, pulmonary fibrosis and thrombotic thrombocytopenic purpura. Our research will certainly lead to conference presentations and peer reviewed publications. In addition, we anticipate that our research will identify new treatment options for the named diseases which currently have an unmet clinical need.

Who or what will benefit from these outputs, and how?

Our disease targets include cystic fibrosis, pulmonary alveolar proteinosis, alpha-1 antitrypsin deficiency, interstitial pulmonary fibrosis and thrombotic thrombocytopenic purpura. We anticipate, that these patient cohorts will benefit, if our translational research programme is successful. More specifically:

Short-term: Proof-of-concept that gene and cell therapy can improve or stabilise disease in our pre- clinical disease models.

Medium term: Translation of gene and cell therapies into clinical trials.

Long-term: Licensing and adopting of gene and cell therapy-based treatments for disease with unmet clinical needs.

How will you look to maximise the outputs of this work?

Collaboration: Approximately 20 years ago we founded a consortium consisting of scientists and clinicians across several UK Universities. We form the largest group worldwide involved in developing advanced therapeutics for pulmonary diseases.

We disseminate new knowledge through interaction with academics at conferences and publications. Importantly, we publish all results regardless if they are proving or disproving our hypothesis.

In addition we are actively involved in patient and public education around advanced therapeutics.

Species and numbers of animals expected to be used

- Mice: Over 5 years we anticipate to use approximately 5000 mice
- Rats: Over 5 years we anticipate to use approximately 100 rats

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Over 95% of animals used in our studies are normal healthy mice or mice in which disease is induced. The vast majority of mice used will be treated when more than 6 weeks old. We anticipate that advanced therapeutics for the disease indications that we are describing in this license will be administered to adult populations rather than children. Mice > 6 weeks old therefore mimic the intended age of the proposed patient cohorts.

Mice are used because relevant disease models have been generated through our ability modify and shut-down genes.

Occasionally, but very infrequently, we confirm data generated in rats. This is particularly relevant for research related to cystic fibrosis. CF knockout mice do not develop CF lung disease, whereas CF rats develop characteristic pulmonary features of CF.

Typically, what will be done to an animal used in your project?

A typical experiment (see more details below) includes administration of a gene transfer agent or of gene modified cells to the lungs of mice using non-invasive delivery methods via nose or mouth. At specific time-points we may collect small volumes of blood via the tail vein or will collect urine. In some animals we may collect breath under anaesthesia by putting mice into a breath collection chamber or we may image gene expression in anaesthetised mice. Most animals (~90%) are humanely killed within a few months post gene/cell transfer. A small number of animals is followed up for longer periods (max 24 months) to assess duration and impact of the treatments on the disease . In addition to wildtype mice, disease-specific genetically modified mice may be used. In a typical experiment animals will be treated with an advanced therapeutic once and culled at a specific time-point post treatment.

Please see below specific details describing the typical protocol for mice and rats on this license.

Life-history: typical scenario protocol for mice

1. Determination of destruction of specific genes (used to generate a model with lung disease). This will for example include taking an ear punch from the animal.

2. Anaesthesia (not more than 12 times over at least 6 months)

3. Administration of the novel drug to the nose, which is then "inhaled" into the lung (animals may receive 1 to 12 doses over 6 months)

4. Blood and urine sampling (typically 6-10 collections over 6-12 months)

5. Collection of breath under recover anaesthesia and/or lung function measurement under terminal anaesthesia (typically 1 measurement)

6. Humane killing

Proportion of mice/rats treated using the typical protocol: >90%

Severity limit of the typical protocol: mild

Some animals (<5%) may be housed and observed for up to 24 months

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority (>95%) of our procedures are classified as sub-threshold or mild and do not induce pain or discomfort. Occasionally animals show signs of being unwell for a few hours after treatment (most often this will be visible by looking at the fur, with raised hair (piloerection) being a common sign. The need for human killing of animals post-treatment due to side-effects is extremely rare.

The impact of a typical experiments described above may include a slight and transient discomfort while collecting an ear punch. Gene transfer to the lung may induce transient local inflammation, which generally resolves within 24 hours after treatment and may result in raised hair. Blood sampling via a tail vein may cause transient discomfort due to needle prick. Collection of breath and imaging protocols will be performed under anaesthesia.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice:

95% mild, 5% moderate

Rats:

100% mild

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We aim to develop advanced therapeutics, namely gene and cell therapy-based treatments for pulmonary and non-pulmonary diseases by using topical delivery of gene and cell therapy products to the lungs. Many diseases such as cystic fibrosis, pulmonary alveolar proteinosis, alpha-1 antitrypsin deficiency, interstitial pulmonary fibrosis and thrombotic thrombocytopenic purpura have significant unmet clinical needs. Gene and cell therapies have been successfully developed for several diseases indications and offer promise for others.

As part of our translational research programme we have to assess efficacy and duration of gene expression, as well as toxicity in relevant rodent disease models. Assessment of transduction efficiency and duration of expression requires all cell types that our vector transduces (eg ciliated airway epithelial cells, goblet cells, Club cells, basal cells and pneumocytes) as well beating cilia to be present. Assessment of toxicity and inflammatory responses requires a model in which inflammatory cells such as neutrophils, lymphocytes and macrophages can migrate from the bloodstream into the lungs in response to a challenge such as vector administration. These conditions are only provided in living animals.

Which non-animal alternatives did you consider for use in this project?

In parallel to work in mice we conduct studies in human air liquid interface cultures, human precision cut lung slices and in ex vivo lung perfusion models.

Why were they not suitable?

However these human ex vivo models do not have an intact immune system which impacts on toxicity and efficacy and also don't allow us to assess changes in relevant disease biomarkers.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The projected number of animals in the license reflects the number necessary to achieve the scientific objectives outlined in the programme of work and covers development of new therapies for a number of diseases with unmet clinical needs. The numbers are also based on our previous experience generated over the last 10 years as part of 2 previous project licenses and estimates of the requirements of translational research studies by us and scientists. Projected n numbers also reflect requirements of the regulators (eg bodies such as the Medicines and Healthcare products Regulatory Agency (MHRA)).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have over 25 years experience designing mouse experiments and always aim to minimise n numbers as much as possible through:

- randomisation

- validation of endpoints and biomarkers in pilot experiments to generate baseline data for subsequent power calculations.

- careful selection of endpoints and biomarkers to reduce variability and hence n numbers

- use of the NC3R EDA tool

- ensure that operators are trained to high standards

- careful planning and discussion of experiments to ensure all appropriate control groups are included in the design

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We routinely assess and adapt the size and output of our breeding colonies.

As mentioned above we conduct pilot studies to generate data for subsequent power calculations.

We carefully archive materials from control animals (eg blood, DNA, other body fluids) and reuse this material during analysis of experiments where appropriate.

We use tissues as efficiently as possible (for example by tying of the bronchus to the left lung for RNA extraction before fixing the right lung for histology)

We frequently use a histology core facility to ensure optimal processing, cutting and staining of tissue sections.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will mainly use wildtype mice and genetically modified murine disease models. AATD, TTP and PAP mouse models are mild, breed normally and do not show outward signs of disease. CF knockout mice frequently suffer from intestinal disease leading to high post-weaning mortality. However, we have in the past and will continue to use refined CF models in which the intestinal disease has been corrected through more sophisticated molecular biology strategies.

Gene transfer is performed through topical non-invasive administration to the mouse lung and sometimes through intravenous injections or nebulisation. These methods are minimally invasive.

In some experiment wildtype rats may be used to confirm studies in a larger rodent model.

Why can’t you use animals that are less sentient?

Mouse models are the most commonly used model for gene transfer studies. Although the lung structure and cell composition is not identical to human lung, mice present the best low ranking species for these studies.

We can't terminally anaesthetise mice because for assessment of efficacy and toxicity after gene and cell therapy, the animal has to be alive for indicated times post-dosing.

Some of our gene transfer agents achieve gene expression for prolonged periods of time (sometimes for the life-time of the animal after a single dose). Long-term follow up of efficacy and toxicity is therefore necessary in some experiments.

On rare occasions we may conduct gene transfer in neonates to mimic treatments of babies/children in which the organs are still growing.

In contrast to CF knockout mice, CF knockout rats develop certain features of CF lung disease. We will use wildtpye rats in some experiments to characterise the expression profiles of our advanced therapeutics in rats to allow future studies in CF knockout rats.

Non-protected animals (e.g. invertebrates such as insects, decapods, nematodes) or less sentient animals (eg zebrafish) cannot be used to meet some or all of your objectives of our studies largely. We require a model with lung structure and cell composition as well as the immune system that closely mimic human.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

If required for some experiments we may increase post-treatment monitoring and post-treatment care.

Our experiments are generally mild and do not require any post-operative pain management. However we will seek advice from experts if pain management is required.

In our experience provision of wet food in the cages enhances post-treatment care following general anaesthesia and we provide wet food.

In regular meetings, we also share experiences in animal handling amongst the team to ensure everybody uses the most advanced methods

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are several best practise guidelines that are published and that we are fully aware off and will adhere to. Some examples are listed here: www.aaalac.org/resources;www.reference- resourcesarchanimaltraining.com; (https://norecopa.no/PREPARE).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will carefully review all information communicated by the home office and establishment staff/relevant named persons as well as our establishments 3Rs group. In addition we will keep updated with the NC3Rs website and relevant other activities.

46. Development of Humanised Mouse Models for Study of Cancer Immunotherapy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Transplantation, Cancer, Treatment, Immunotherapy, Safety

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to study how the immune system recognises cancer cells, and test the safety and effectiveness of drugs that can kill tumour cells by interacting with the immune system.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is one of the leading causes of death in the world. It is now recognised that the immune system has a critical role in how cancers develop, and that drugs that manipulate the immune system have great potential as better therapies. The proposed project will allow a better understanding of how the immune system recognises tumours, and to test the safety and effectiveness of drugs that can kill tumour cells by acting on the immune system. This could lead to development of new therapies that are more effective and cause fewer side effects to patients.

What outputs do you think you will see at the end of this project?

It is expected that the proposed project will ultimately enable novel models of human tumours to be used not only to study cancer biology in vitro, but also to enable drug development and screening. Moreover, it is anticipated that advanced immunotherapies can be developed and optimised that will enable highly effective tumour eradication without the devastating side-effect profile associated with classical chemotherapy agents. The insights generated by this project will be shared through publications to enable other researchers to incorporate the findings into their research programs.

More specifically, we expect to generate at least one new therapy and advance it to a stage that it can be investigated further in large animal models. In the long-term (5-7 years), we expect that the findings of this study will result in the design of at least 1 human clinical trial to test the safety and efficacy of a novel therapy developed in this project.

Who or what will benefit from these outputs, and how?

In the short-term (1-3 years), the primary beneficiaries of the proposed project will be other researchers who are also developing immune-related therapies for treatment of cancers. We anticipate that the findings of this study will be of broad relevance to the research community in this field. In the medium term (3-5 years), we anticipate that industrial companies involved in the manufacture of immunotherapies, who are essential for the ultimate production of these therapies, will also benefit from the findings of this study. In the long-term (5-7 years), this project will benefit patients with cancer. The benefit will initially be limited to those patients enrolled in clinical trials investigating the safety and efficacy of immunotherapies developed in this project. We hope that ultimately (7-10 years) large numbers of patients will benefit from the findings of this study, once the immunotherapies have been shown to be safe and effective in clinical trials and can be manufactured at large scale.

How will you look to maximise the outputs of this work?

We will disseminate all findings of our studies, including unsuccessful approaches, through publication in peer-reviewed journals, presentation at scientific conferences, and through meetings with other researchers. All publications will be open access, including through platforms such as F1000Research. This project includes collaborations with a large

number of researchers with expertise in complementary areas, and this network will be utilised to maximise the dissemination of the new knowledge gained through this project.

Species and numbers of animals expected to be used

- Mice: 3475

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will use adult mice. Mice are the lowest species with a comparable physiology that enables useful information to be gained relating to the safety and effectiveness of human immunotherapies.

Importantly, genetic strains of mice are available that allow studies to be designed that can generate valuable information about specific cellular therapies. Moreover, mice can be used effectively to model the human immune system by injecting them with human immune cells, followed by transplantation with human cancer cells, thus allowing the study of the human immune response to cancer.

Typically, what will be done to an animal used in your project?

In the typical experiment, an adult mouse will receive sublethal irradiation, followed by adoptive transfer of human immune cells through injection into the abdominal cavity. After several (typically 4-

12) weeks later, the animal will be surgically transplanted, on one occasion, with tumour (or control) cells or tissue into the abdomen under general anaesthesia. After 2-4 weeks, the animal will be given an immunotherapy agent by intraperitoneal injection on a number (typically 3-5) occasions over 1-2 weeks. The animal will then be killed by Schedule 1 methods (humane killing of protected animals under the Animal (Scientific Procedures) Act 1986), several (typically 4-6) weeks later. Some animals may undergo non-invasive imaging on a number (typically 3-5) of occasions. The typical animal will undergo one or two surgical procedures and be kept for approximately 16 weeks, when it will be killed electively while still well and without clinical signs. In some cases, the animals will be killed by removal of organs while under deep general anaesthetic.

What are the expected impacts and/or adverse effects for the animals during your project?

It is expected that most (more than 90%) of animals will recover rapidly and well from tumour cell transplantation.

Injections with immune cells (such as white blood cells) is also generally well tolerated and most (more than 90%) animals will not experience adverse effects from this procedure.

In some cases, the animals may experience weight loss, reduced food intake, reduce movement or an abnormal coat. In such cases, the animals will be culled if these clinical signs do not respond to treatment (such as high energy and easily digestible diet) and persist for up to 24 hours.

When rapid adverse effects may be expected, animals will be monitored very frequently (up to one hourly) during the initial period (~6 hours) when adverse effects are most likely to occur (based on data from previous animal experiments and clinical studies).

Animals will also be culled if they experience clinical signs that approach the limits described in the project according to the Home Office guidelines.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 65% of animals will undergo a surgical procedure and will therefore experience moderate severity, or experience moderate severity as consequence of administration of immunotherapies. Approximately 25% of animals will only receive cells by intraperitoneal or intravenous injection and will experience mild severity. The remainder (10%) may be culled for tissue and will experience subthreshold severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The definitive examination of the efficacy and safety of anti-cancer drugs requires examination in intact animals, including those with a competent immune system. This is a necessary and pre-requisite ‘final’ step for the clinical translation of these cancer therapies and cannot be completed without animal experiments. Much of the proposed work is carried in the laboratory and using human tissue only, thus minimising the need for animal experimentation. Importantly, it is anticipated that this work will lead to the refinement and

optimisation of laboratory models of cancer which can be ultimately used to replace experimental use of animals.

Which non-animal alternatives did you consider for use in this project?

We have 3 key strategies for the development and use of non-animal alternatives.

1. We intend to use humans cancer cells and tissue in the vast majority (>95%) of our studies. Animal cells will only be used if they are necessary control for the use of human cells, or where equivalent human cell alternatives is not available. We therefore anticipate using very few animals for the generation of tumour cells.

2. We will continue to make extensive use of sophisticated human cell culture systems, such as organoids and tumoroids technology, to study the safety, functions and immune response to cancer in vitro.

3. We have developed mechanism for perfusing human organs ex vivo, on a specialised machine, for prolonged periods in order to test the functions of transplanted human cells and tissue.

Why were they not suitable?

We are using all of these three alternative approaches to reduce the number of animals used in the proposed experiment. However, the definitive investigation of the immune response to tumours, and the safety and efficacy of immunotherapies requires an intact and functional immune system in an animal model.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals have been estimated based on the range of studies that are planned, as well as based on the previous similar studies we performed during the last five years. Based on our previous experience, we are able to predict, for each study, the number of animals that are required to generate reliable and reproducible data. Using our previous experience, we are also able to predict how many studies we can perform in a given time period.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have generated a wealth of data from previous experiments that enable us to estimate the anticipated effect size and variation in the experimental data. This data will be used to ensure appropriate experimental group sizes. We routinely randomise animals to experimental and treatment groups and all experiments are conducted and/or data analysed in a blinded manner to reduce bias. When a new tumour cell or immunotherapy is under investigation, we will first perform pilot experiments with small animal groups (typically 2-3 animal per group) to confirm the appropriateness of the experimental design and to generate pilot data to enable group sizes to be formally calculated. We will also use tools such as the NC3Rs Experimental Design Assistant to ensure experiments are appropriately planned to generate reliable and reproducible data. We will also take into consideration any regulatory requirements relating to the reproducibility of the data, in order to ensure data generated from this study is suitable for informing design of future clinical trials.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continue to reduce the number of animals we use through a number of simultaneous strategies:

1) We will share excess breeding animals with other researchers and use any animals culled as tissue donors for use by our group or by other researchers.

2) Where possible, we will use each animal as its own control, for example by transplanting control and tumour cells in two separate sites (for example, into each kidney) in the same animal. This will reduce inter-animal variation and reduce the number of animals used.

3) By generating large quantities of human cells, where appropriate, we can continue to perform new studies using the same human tumour, which reduces the variation associated with different human donors and reduce the number of animals used.

4) By monitoring animals for prolonged durations, and through the use of non-invasive monitoring techniques (such as imaging), we can generate longitudinal data about tumours and immunotherapies without the need to cull animals at numerous timepoints.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The experimental models and techniques used in this project can be broadly divided into three groups:

1) Transplantation of tumour or control cells or tissue into animals to assess the tumour biology

2) Reconstitution of the animals with a human immune system to assess immune response to the tumours

3) Administration of immunotherapies to assess their safety and efficacy in reducing tumour growth

All experimental models have been refined to ensure they cause the least pain and suffering. Importantly, none of the procedures are expected to result in severe clinical signs (such as persistent abnormal behaviour or persistent weight loss). Animals will be culled if they display clinical signs that do not respond to treatment (such as easily digestible food or pain relief medication). Animals therefore will not be permitted to experience lasting harm.

Why can’t you use animals that are less sentient?

Mice are the least sentient animals that can be used to generate valuable data to investigate the therapeutic potential of human immunotherapies. As the immune response to tumours, and the action of immunotherapies takes days to weeks to manifest, experiments cannot be performed exclusively under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In close collaboration with the staff at our animal facility, we have a robust mechanism for the post- operative monitoring of our experimental animals. Animals are also given routine post-operative pain relief medication, which has been proven to be effective in previous similar studies. When adverse effects may be expected, we readily increase the frequency of monitoring to identify animals that may be experiencing adverse effects. We also have our own dedicated animal technician who ensures that our animals receive close attention if there are any concerns. We have achieved a number of significant refinements during the previous series of studies. These include enhanced environmental enrichment (such as extra card-board housing) and use of high-energy or tasty diets to prevent weight loss, improved techniques for transplantation of cells in the kidney or abdomen (such as using special needles to shorten the duration of the procedure). Where adverse effects may be expected, we will perform particularly close and frequent monitoring of animals, including

through the use of observation sheets and body weight records. particularly where. We will continue to strive to develop new refinements.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All experiments will be conducted and reported in adherence to best practice guidelines including those published by the Laboratory Animal Science Association (LASA), such as guidelines for record keeping, performing surgery, education and training, and reporting of experimental results. We will also follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines on experimental conduct including study design, randomisation, avoiding bias and statistical analysis of results.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As a current project license holder, I am closely involved with the activities of the 3Rs committee at my institution, including the development of recommendations and dissemination of information relating to advances in 3Rs. I intend to continue with my activities, including through review of relevant publications, guidelines and best-practice information.

47. Modulation of Synaptic Transmission in Mammalian Central Nervous System

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

synapses, interneurones, hippocampus, cerebral cortex, mental health

Animal types	Life stages
Rats	adult, pregnant
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this continuing project is to study the properties of neuronal synaptic connections in the brain, understand their roles in the circuitry and characterise modulators of these connections.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cortical regions contain millions of neurones that are connected to each other through millions of synapses. These connections involve different types of cells that have distinct properties and roles in the circuitry. Understanding how neurones are connected to each other in healthy animals and how specific drugs modulate these connections are ultimately key elements to building information towards understanding pathological behaviours and their treatments.

What outputs do you think you will see at the end of this project?

This project will further our understanding on how the brain processes information and produce data that is of importance to a large number of basic and clinical scientific questions. The work described here identifies the basic building blocks of this circuitry, how its dynamic properties shape information as it is processed, how the exquisite precision observed in adult brain develops and how drugs can induce minor changes to this connectivity and result in a change in the brain response after a prolonged treatment. The in-depth studies of brain regions outlined here will provide many other scientists with the circuit components needed for an understanding of how the brain acquires, processes and stores information and what may go wrong in disease, while the recording of brain connectivity and morphological data will be widely used by computational neuroscientists in the construction of models that explore information processing without further use of animals. Information on the effect of modulators of brain connections will also allow us to understand changes that occur in the brain and are responsible for the reduced reaction to certain drugs after repeated use. All data will be presented at national and international conferences and published in scientific journals.

Who or what will benefit from these outputs, and how?

These outputs will benefit to:

- Scientists/doctors studying brain connectivity and its implications in disease states.

- Computational scientists who will use the outputs from this project to build realistic models based on experimental data.

- Information from this project will also be useful for patients who suffer from mental health disorders and scientists who are studying the causes and treatments of these conditions. This project will give invaluable information on the appearance of tolerance following treatment and potential new therapeutic avenues to prevent it.

How will you look to maximise the outputs of this work?

All data will be presented in national and international conferences, discussed with experts in the field and published in scientific peer-reviewed journals. Follow-up funding based on the data collected will be sought.

Species and numbers of animals expected to be used

- Mice: 280
- Rats: 260

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All experiments outlined in this licence will be performed on either adult rats or mice. The basic brain circuits of rodents appear to be sufficiently similar in all mammalian species to allow fundamental scientific questions relating to the human brain to be addressed. A large part of the data that has been obtained in our laboratory and other across the world for most of the past century comes from small rodents. The work that is presented here will allow comparisons and further advance our understanding of brain connectivity. The use of adult rodents will allow the study of a fully developed brain and the potential change in connectivity using drugs that are used in patients.

Typically, what will be done to an animal used in your project?

Some animals will be terminally anaesthetised, perfused with artificial cerebro-spinal fluid to remove the blood and allow better analysis of the data and brains will be removed. Brain slices will then be obtained for recording of neuronal activity and further analysis.

In some experiments, mice will be injected twice a day with modulators of neuronal connectivity for up to one month. They will then be terminally anaesthetised and perfused prior to the removal of their brains. Behaviour experiments will be performed before injection, then once weekly to monitor tolerance and before the culling of the animals.

What are the expected impacts and/or adverse effects for the animals during your project?

The side effects expected in the animals will be mild. We will be looking to use minimally-effective doses of drugs that will lead to tolerance in the animals with minimal side effects. Due to the drugs used, animals will be mildly sedated. However, abnormal behaviour is not expected with the drugs and doses that will used in this project.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The protocol described in this license is of mild severity overall with a large number of the animals following a non-recovery protocol. The injections may lead to mild side effects, however, these will be of short duration and will not affect the behaviour of the animals. Rodents will be culled either by a schedule I method or under terminal anaesthesia. Each licensee will be expected to obtain the Home Office PIL A/B and be trained appropriately. Supervision will be in place until the licensee is deemed competent in practicing the procedures described in the protocol minimising animal suffering.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of animals is essential for obtaining data that will increase our understanding of information processing, memory and behaviour in absence of disease. The relationship between role and functions of cells in the brain, their response to drugs and brain anatomy can only be explored in living tissue.

This will allow existing and new data to be linked, compared directly and the more complete picture that emerges to contribute to models of brain cells and circuits. These models/circuits can provide invaluable insight when the questions addressed become too complex. The varied nature of our data will allow some parameters to be used to build the models (e.g. highly detailed anatomy of brain cells) and others (such as the properties of the connections between these cells) to be used to ensure that these models behave appropriately. The preservation of these valuable data will prevent repetition of experiments that are costly in time and expertise as well, importantly, as in animal use.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives considered included the use of immortal cells and computational modelling.

Why were they not suitable?

Cultures of immortal cell lines will be used to answer some of the questions set in this license on how the connections between brain cells are formed. Most of our knowledge of how these connections are altered in many neurological disorders has been extrapolated from the research done using animal models that have different brain anatomy and display different behaviours. However, a number of mutations discovered in humans do not in fact cause disease states when introduced into rodents. The potential impact, both in basic

research and medicine development, of model systems based on human-derived neuronal tissue that is able to transform into a cell type of interest is therefore substantial. In parallel to the cultures of immortal cell lines, a new co-culture model system which will allow us to 'build' the human inhibitory synapses 'in a dish', and study how individual mutations discovered in patients affect this process. This is an important step towards understanding how the connections are formed and test new therapeutic targets. However, cell cultures do not replicate the entire biological system and will only give limited information. The characterisation of neuronal connectivity and modulation of the transmission of information following therapeutic treatment associated with some change in animal behaviour will ultimately require the use of animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

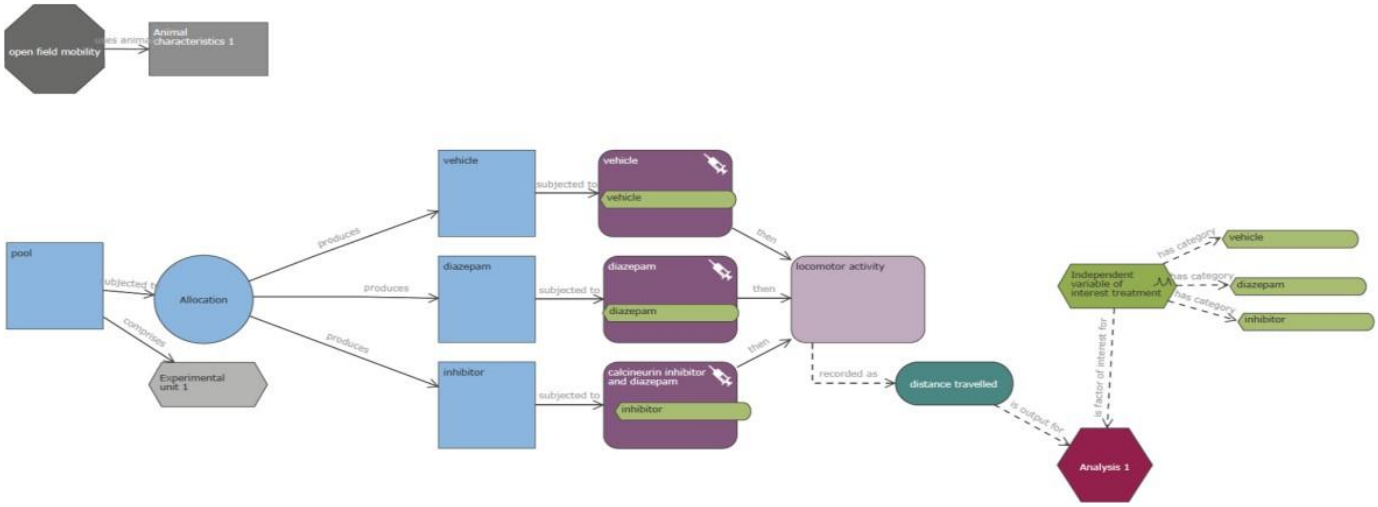
The number of animals needed for this project was estimated using previous knowledge and experience in neuroscience research. Previous experiments allowed us to predict the amount of variability between experiments and to estimate the minimal number of animals that will be required to obtain a clear answer to the research question/objective using power calculations. Typically, each animal used will provide a large amount of data towards more than one objective.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Power calculations were used to predict the number of animals needed to achieve all the objectives on this licence. Advice taken from local statisticians and the use of the results from preliminary experiments obtained on another licence helped in the design of future experiments. The amount of data/information gathered per animal in one single experiment will be maximised in order to reduce the number of animals to be used in this project and answer all the questions set in each objective.

Negative results will also be reported to prevent unnecessary studies to be conducted by other laboratories. Allocation to treatment groups will be randomised and all experiments and analyses will be blinded to maximise the statistical power of each mice used in the study.

Example of experimental design



What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Training of all research staff will be compulsory for all the techniques used in this project to ensure good practice and reproducibility of the results. The use of brain tissues (from each experimental group) will be maximised to increase the amount of data collected from each experiment. Pilot studies with few animals will be carried out for each new drug to be tested to ensure that the best dose regimen is used and animal welfare is appropriately addressed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Although primates are closer to humans in behaviour and brain formation, the basic brain circuits of rodents appear to be sufficiently similar in all mammalian species to allow fundamental scientific questions relating to the human brain to be addressed. A large part of the data in our archive, like much of what has been obtained in other laboratories across the world for most of the past century, comes from rats or mice. The data obtained in this licence will use the same species and similar protocols to allow existing and new data to be compared directly and the more complete picture that emerges to contribute to models

of neuronal circuits. We will use healthy adult rodents to provide a detailed study of brain circuits in the absence of disease and the methods used will be of mild severity.

Why can’t you use animals that are less sentient?

All animals used in this licence will be adults to allow the neuronal circuitry to fully develop and drugs to be tested on an 'adult' model. Animals that have been terminally anaesthetised will be used to obtain data towards some of the objectives of this licence.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We consider the welfare of the animals of paramount importance and the procedures will be regularly reviewed for potential refinement. Single housing will not be considered in this project and rodents will be provided with high quality bedding material. We do not expect any complications following treatment of the animals. However, pilot studies will dictate appropriate doses and animals will be closely monitored. Animals will be handled with tunnels or cupping, acclimated to their environment before the behavioural testing and trained appropriately according to the test used.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will ensure that experiments are conducted in the most refined way by following standard operating procedures from the Home Office and institution and the guidelines for the use of animals [Animal Behaviour (2018)].

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

My link to the NC3Rs through the newsletter and social media allows me to keep up-to-date with any advances in this domain. Updates from the NC3R regional manager during the AWERB meetings will also inform me of new information on experimental design, good practice and replacement methods. Any new development of new 3Rs technologies related to this project will be scrutinised, implemented if possible and shared with NC3RS regional manager and community.

48. The Testing of Bone Scaffolds in Sheep

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

bone repair, scaffold, sheep

Animal types	Life stages
Sheep	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

In this study we will test the ability of a biodegradable polymer based scaffold to encourage bone growth. This will be tested in a bony defect in the knee of sheep. The material to be tested is a 3- dimensional (3D) scaffold with a high number of linked pores, which should allow cells to migrate through the defect whilst providing support for the bone to grow. In addition, the scaffold will be coated in substances which encourage bone to grow which should increase the rate of bone growth.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Worldwide there are a large number of surgeries carried out that require bone grafting, even though there are significant risks associated with it, for example rejection and infection, multiple surgeries, increased hospital stays and greater demand and costs put on the NHS. There is also usually a lack of sufficient bone graft, therefore donor bone may be needed. The materials we are working with will hopefully be able to support bone growth, cell migration and the formation of blood vessels within a defect site, and could benefit humans. This in turn should reduce the number of surgeries and infections associated with bone grafting and provide a material which encourages better bone fill to a defect site.

What outputs do you think you will see at the end of this project?

Data will be used to create publications which will be submitted to high impact factor peer-reviewed journals. In addition the data will be presented at international conferences.

The aim of this period of funding will be to develop a biodegradable biomaterial implant which is capable of promoting and inducing bone regeneration and vascularisation in bone defects. Hopefully removing the need for several surgeries, an increased recovery time and the risk of donor-site morbidity.

Who or what will benefit from these outputs, and how?

The data derived from this project will be used by scientific researchers, industrial partners, clinicians and veterinarians.

Short term benefits (1 - 3 years) - information shared through peer-reviewed journals and international conferences

Medium term benefits (3 - 5 years) - successful regulatory approval of the material; GMP manufacturing, packaging and storage

Long term benefits (5 - 10 years) - phase 1 clinical trials; reduction in the use of autograft and therefore the associated complications and the replacement of products on the market which have proved imperfect or require several surgeries

How will you look to maximise the outputs of this work?

We will be collaborating with different research groups and any industrial partners to develop our technology further. In addition, we will be publishing the results of this study as well as presenting our findings in symposiums and conferences

Species and numbers of animals expected to be used

- Sheep: 24

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

Cell based studies are useful to look at how cells will behave in contact with the scaffold, however cell assays alone cannot adequately model the complete array of effects important in bone modelling or repair. Using stem cells we have shown that cells were viable, proliferated, and were evenly distributed throughout the scaffold, suggesting that the material would not be toxic and encourage bone growth.

Predictions of degradation rates and pore numbers have been obtained from these studies for varying formulations; however none of these assays can adequately model the in vivo environment of a whole animal. Therefore we need to test the material in an animal model before it can be translated into use in a human.

The animal to be used is a skeletally mature female sheep as they have a similar body size and rate of bone remodelling as humans.

Typically, what will be done to an animal used in your project?

The model is a bone defect with a maximum width of 11 mm x 15 mm height that is drilled into the medial femoral condyle of both hind legs as this reduces the number of animals used in the study as each leg acts as a separate sample. We have previously shown that the sheep tolerate bilateral surgery very well. We do not see animals remaining recumbent and unable to stand post-operatively after creating defects in both hind legs (therefore 2 defects per sheep). The sheep are slightly lame for up to three days when compared to un-operated sheep, but are then typically 100% sound with no effect on gait with a return to pre-operative normality. This is evident by the fact that sheep have previously been able to stand on their hind legs shortly after surgery in an attempt to reach food, showing that the analgesia is highly effective. The gold standard for bone grafting is the use of autograft, which is the positive control in this study. Bone trephines have been designed to allow sufficient bone cores to be collected from two femoral condyles in a single sheep to fill one defect with bone graft, therefore removing the need for bone harvesting from the iliac crest. An empty defect is used as the negative control.

The animals will be housed indoors in small pens for a week post-surgery and monitored clinically for any signs of pain following injection and then allowed to roam free in a field/barn for the remainder of the study during which further activity (e.g. Fitbark) and clinical monitoring will be undertaken.

At the end of the study, animals will be euthanised and when dead the femoral condyles will be removed and fixed for a period of at least one week. Defects will then be scanned using micro- computed tomography (micro-CT) before carrying out histology and immunohistochemistry to assess bone quality and cell types.

What are the expected impacts and/or adverse effects for the animals during your project?

The overall severity of this procedure is moderate. We expect that the animals will be slightly lame for a couple of days and then will have no adverse effects of the surgery. The animal may show mild signs associated with recovery from general anaesthetic, such as appearing slightly ataxic and sedated, but this is transitory and our experience is that sheep are usually standing within 15 minutes of the end of the general anaesthetic. Analgesia will be given for 72 hours post-operatively, unless an animal is assessed by the NVS to require additional pain relief.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

During this protocol 100% of the animals will suffer moderate levels of severity as a result of the surgical procedures conducted under general anaesthesia.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In vitro studies are helpful as a first stage test to look at cell proliferation, cell differentiation, toxicity and cytocompatibility. However, in vitro assays alone cannot adequately model the complete array of effects important in bone and cartilage modelling or repair. Previous work has been carried out looking at the growth of mesenchymal stem cells on the product. This study showed that the cells were viable, proliferated, and were evenly distributed throughout the scaffold. Degradation and porosity studies were investigated by submerging samples in saline solution. Predictions of degradation rates and porosities have been obtained from these studies for varying formulations; however none of these assays can adequately model the in vivo environment of a whole animal.

All products will have been thoroughly tested in vitro for biocompatibility and cytotoxicity before delivery into the sheep. The next stage of the study is to demonstrate the bone tissue’s response to the scaffold material. Therefore, we plan to use a bone defect sheep model to look at ingrowth into the product.

Which non-animal alternatives did you consider for use in this project?

In vitro testing was carried out to assess the biocompatibility and cytotoxicity of the material, but it is not possible to recreate the in vivo bone environment.

Why were they not suitable?

It is not possible to achieve the aim of this project without implanting the material directly into a bony defect within a living animal, as in vitro approaches cannot, to date, mimic the complex physiological in vivo microenvironment involved in tissue repair.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We will always seek to use the minimum number of animals necessary to achieve the objectives of the project whilst maintaining statistical validity of results. We have based the numbers of sheep required on previous studies we have carried out and that we have published in the literature. In this project we will be creating two defects per sheep, one in each hind leg, which allows us to reduce the animal numbers used per study. We will be using a minimum of 7 defects per experimental and control group.

In our research we will randomise our experiments to avoid bias, for example we randomise which animals get which treatment i.e. biomaterial or control and in what order they undergo procedures. We will analyse our data in a blinded fashion and use coding systems to anonymise data, thereby reducing unconscious bias. In some instances we may ask our collaborators to randomly allocate animals to experimental groups and to retain the information as to which group and animal belongs until the final results are collected. We believe that these methods contribute to the robustness of our data interpretation. We are committed to working to the PREPARE guidelines.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The NC3R Experimental Design Assistant was used to create a study plan. This allowed us to critique the plan and run a power calculation. Values from our previous studies (amount of bone formation) were used in the EDA and also PS Power and Sample Size (version 3). Setting the power of the experiment at 80%, $p<0.05$ and a standard deviation of 8 we require a minimum of 7 defects per group. At a power of 90% the number of defects required is 8.

We will also maximise the data obtained from each sheep e.g. activity data, micro-CT data, histological and immunohistochemistry data.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

At the end of the study any unwanted tissue may be harvested by other groups for further research.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The drawback of utilising small animal orthopaedic models, for example mice, lies in the inherent differences in terms of bone composition, bone density, and quality when compared to humans. These models are therefore not suitable for evaluating products close to the human market as results cannot be directly extrapolated to human conditions. For this reason we have chosen a larger species, sheep, which is more analogous to humans with a similar pattern of bone ingrowth into porous implants over time. Sheep are placid and easily handled with a similar body weight to humans. They have been used in the study of numerous musculoskeletal conditions and diseases including biomaterial evaluation. Sheep have cancellous and cortical bone, undergo bone remodelling and have a similar healing rate as humans, but they also have plexiform bone (akin to woven bone) and fewer Haversian canals, with differences in bone composition and fracture stress levels. Even though the rabbit is the most commonly used species (approximately 35% of all musculoskeletal research) it shows the least similarities to human bone, with gross differences in bone anatomy in comparison to humans and have a faster skeletal change and bone turnover.

Why can’t you use animals that are less sentient?

Cell based studies are useful to look at how cells will behave in contact with the scaffold, however cell assays alone cannot adequately model the complete array of effects important in bone modelling or repair. Predictions of degradation rates and pore numbers have been obtained from these studies for varying formulations; however none of these assays can adequately model the in vivo environment of a whole animal. Therefore is not possible to achieve the aim of this project without using animals as the only authentic test

of a product designed to encourage bone formation is to place it into challenging bone defects and quantify its effectiveness at encouraging bone formation, and we need to test the material in an animal model before it can be translated into use in a human.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Sheep will undergo only 1 surgical event to create bone defects in each hind leg within the femoral condyle. This allows us to use fewer animals in a study, and we have also previously shown that the sheep tolerate the double defects very well. The region is low weight bearing, not within the joint and previously we have had no issues with significant lameness in any sheep. All surgery will be carried out aseptically in dedicated facilities, according to the LASA guiding principles for preparing for and undertaking aseptic surgery.

The sheep will receive analgesia (e.g. fentanyl patches), as advised by the NVS, pre-operatively and post-operatively to allow for a continuous supply of analgesia for an appropriate period. If for unforeseen circumstances the surgery must be cancelled the patches will be removed and the sheep returned to the flock. Sheep will not then be re-patched and starved for a minimum of 72 hours. In addition, the sheep will receive local anaesthetic in the injection/incision site. To reduce the risk of infection the sheep will receive antibiotics pre-surgery and post-surgery. Non-steroidal anti- inflammatories will be used for up to 72 hours after injection, with the initial dose given before first incision. Animals will be housed in dedicated recovery accommodation post-surgery for 24 hours (or until recovered from the immediate effects of the anaesthesia) and then returned to either the barn or the field for group housing. The decision to transfer the animal from one housing regime to another is dependent upon recovery from the surgery and is therefore made on an individual basis for a particular animal.

There are three clinical score sheets to guide us when monitoring the animals. One covers the mobility, food intake and water consumption, fever, demeanour and respiratory rate and is routinely used for all animals for a minimum of 3 days post-operatively. There is a posture and locomotion scale that is used to assess the levels of lameness and we use the grimace scale to assess levels of pain in the sheep. If any sheep is not recovering as expected, enhanced monitoring will be carried out and the NVS contacted for advice.

After the injectable anaesthetic agents are given all sheep must be intubated for bone defect creation. Sheep are at a high risk of aspiration pneumonia due to a large volume of rumen contents and saliva, but this risk is reduced after the cuff is inflated in the endotracheal tube in addition to tilting the surgical table. Generally the larynx is sprayed with a local anaesthetic agent, for example lidocaine, before the lubricated endotracheal tube is placed using a laryngoscope. This is a challenging procedure in a sheep as visualisation of the airway is difficult due to the narrow opening of the mouth. If intubation is not possible the sheep may be recovered, but strict criteria must be followed to ensure the animal is a viable candidate for recovery and re-use after NVS assessment. No

obvious damage must be seen at the back of the throat and minimal rumen contents should be present in the mouth. A maximum of 6 intubation attempts, over a 30 minute duration, will be made to place the tube. The sheep must

recover properly and be well immediately afterwards. Antibiotics may be given as a prophylactic for aspiration pneumonia. The NVS will be contacted for advice at the time and will then assess the sheep before another anaesthetic event. The animal will be allowed to recover for a minimum of 7 days after a failed intubation procedure. Only one failed anaesthetic attempt and recovery allowed per animal, therefore if the second anaesthetic event (6 intubations) is unsuccessful, the sheep with be humanely killed.

Delivery of any material to a defect site will be closely monitored to ensure that there is not significant inflammation. If inflammation occurs the sheep will be carefully monitored and graded using a locomotion scoring system after consultation with the NVS. Any severe soft tissue inflammation will be treated with antibiotics and local pain relief provided to the animal. If this has not improved the animal's condition over 24 hours then the NVS will be consulted regarding the short term use of an NSAID. If the sheep is significantly lame on both hind legs and is not responding to additional analgesia it will be killed.

During a study animals may be injected with fluorochromes, at 2 known time points for example 19 and 24 weeks post-operatively, to allow assessment of the rate of bone growth within the defect. The stain binds to calcium ions on the mineralisation front and stabilises after 24 – 36 hours after injection. This was used in previous studies under PPL 40/3599 and no adverse effects were seen.

An activity monitor (e.g. Fitbarks) may be worn by animals for either part or all of the duration of a study. The monitors allow us to continuously record the ‘play’, ‘rest’ and ‘activity’ of the sheep before and during the experiment. This allows us to identify early indicators of reduced welfare in the animals and to intervene accordingly. If reduced activity or play and increased rest is noted, the NVS will be asked to examine the animals to identify the cause of the reduced mobility.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All surgery will be carried out aseptically in dedicated facilities, according to the LASA guiding principles for preparing for and undertaking aseptic surgery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

During the study we will be in contact with the NACWO and the NVS in addition to monitoring any updates from the NC3Rs website.

49. Central Nervous System Repair by Cell Transplants

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:

(i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

CNS injuries, cell therapy, repair, transplantation, regeneration

Animal types	Life stages
Rats	adult, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Repair of CNS injuries by transplantation of olfactory ensheathing cells

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Injuries to the brain and spinal cord can lead to devastating and lifelong disability in patients, which also creates an enormous financial burden for families and society. Despite

some advances, there is still no effective treatment. Cell therapy is one of the most promising strategies to restore neurological functions after injury.

The olfactory system is a unique area where the nerve cells in the nose are continually formed through adult life. Their newly formed axons are supported by the special type of glial cells – olfactory ensheathing cells (OECs) and grow from the nose into the brain. Our previous studies have shown that transplantation of OECs into the injured regions of the spinal cord could repair damaged nerve fibres and restore loss of functions. We plan to translate our findings into clinical applications. We will collaborate with clinicians to work on human OECs and test the cell functions in our established experimental models. The outcome of the study will provide important and valuable information for future clinical applications.

What outputs do you think you will see at the end of this project?

The objective is to establish a standardised protocol of the therapeutic cell production and surgical procedure that will be quintessential requirements for OEC transplantation to be applied as a treatment of human brain and spinal cord injuries.

Who or what will benefit from these outputs, and how?

Short term benefits will be to understand and compare the composition of olfactory cultures from the rat and humans and help to develop standardised cell preparation protocols and transplantation procedures which would be applied as a clinical guideline.

Medium-term, our published studies have shown transplantation of rat OECs into our experimental models can repair damaged nerve fibres and restore loss of functions. We now need to compare whether the transplantation of human OECs will have the same reparative ability.

The ultimate benefit of these studies will be the ability to repair central nervous system injuries in man.

Improving nerve regeneration in injured patients may improve their limb power, sensation, bladder, and bowel control. Results will be published in peer-reviewed journals to inform other scientists, clinicians, and patients.

Future applications in patients may extend beyond spinal cord and root injuries to include stroke, head injury, and patients with visual impairment. These treatments will allow the patients to resume a degree of normal life functions and reduce the burden of care on the community.

How will you look to maximise the outputs of this work?

We have been collaborating with researchers and clinicians in the UK and internationally. We frequently communicate with all our collaborators to discuss the research progress and

regularly attend scientific conferences to report our results. Our results are also published in peer-reviewed scientific journals.

Species and numbers of animals expected to be used

- Rats: Total adult: 980; Day 1-8 pups: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rats have been selected for this work, as they are the lowest vertebrate group with a well characterised central nervous system that we can model the human system. Adult rats from three months old are used in this study since the damaged CNS nerves are not able to regenerate spontaneously. We use early postnatal rats (postnatal day 1-8) for the pilot in vitro study since the neural tissues retain viability in culture conditions at this life stage.

Typically, what will be done to an animal used in your project?

All animals will undergo non-invasive behavioural tests pre- and post-surgery; the duration of one session per rat is around 5 minutes, one or two sessions per week for up to six weeks.

Under general anaesthesia, animals receive laminectomy at the cervical or thoracic level and lesion of the spinal dorsal column or the dorsal roots. About 60% of animals will receive the implantation of therapeutic cells. The exposure will be closed by muscle and skin sutures. The duration is up to one hour.

All animals will receive the administration of analgesic agents after surgery. Some will receive immunosuppressive drugs or control substances by intraperitoneal, subcutaneous injection or drinking water under Laboratory Animal Science Association (LASA) guidelines.

At the end of experiments, the animals will be terminally anaesthetised by exposure to carbon dioxide gas in a rising concentration followed by perfusions.

What are the expected impacts and/or adverse effects for the animals during your project?

All the rats will undergo surgery. Post-surgery pain will be mitigated by the administration of analgesic drugs during the first week.

The animals may show reduced activity and about 5% weight loss during the first week post-surgery. Some locomotor changes will be present when animals are undergoing

specific tests. Neurological signs may include inflammation of paws and self-mutilation in a small number of cases during the six- weeks experimental period. This happened through past experiences in our studies. We used a mildly aversive tasting substance such as picric acid or paw gloves to ameliorate or prevent this from happening.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate. In all rats with surgical procedures.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In the past decade, alongside the in vivo work, we explored a few in vitro models e.g. culturing neonatal spinal cord slices. However, (1) Failure of CNS axon regeneration occurs in mature age. However, adult neural tissue does not survive robustly in culture; (2) Failure of axon regeneration is not only the intrinsic properties of the neurons but also due to the complex CNS tissue environment; (3) The in vitro models cannot replace the in vivo models for assessing neurological function outcomes;

(4) Surgical procedures for transplantation cannot be simulated on the cultured tissue.

For translating our work to future clinical applications it is essential to investigate the methodology of introducing and incorporating transplanted cells into adult animals so that we can model the human system. Since the criterion of success is the outcome of motor and sensation functions it is not possible to dispense with the animal models.

We will use early postnatal brain slice and spinal culture models alongside the in vivo work as an initial screen to test the therapeutic effects of cell types and numbers on neurite outgrowth before undertaking in vivo experiments to minimise the number of animals used, to reduce and avoid the animal welfare issues associated with surgery and caring for rats with spinal cord injury.

Which non-animal alternatives did you consider for use in this project?

N/A

Why were they not suitable?

N/A

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We plan to have 5 batches of in vitro brain slice assay to screen the efficacy of the cells produced. Eight pups will be used for each batch. Two hundred pups in total will be needed for 5 years.

We plan to use 2 experimental models to test the reparative olfactory cells from rats and humans. Each model needs about 14 rats per batch, 2 batches (n=28) for testing rat cells and 2 batches (n=28) for human cells each year. Around 560 rats would be needed for 5 years. Around 70 rats would be needed for tissue culture work each year, around 350 rats for 5 years. The total number of rats for 5 years would be around 980.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All candidate cells are first tested in vitro for their ability to fasciculate, elongate, and induce axon growth. These in vitro assays are being constantly improved and extended so that they enable a more complete profile of the reparative abilities of the candidate cells to be assembled.

This avoids the use of living animals to establish these cellular properties. The findings of the investigations are subjected to parametric and non-parametric statistical analysis to ensure the use of only the minimal amount of material needed to establish the reparative properties of the cells. It is only once these properties have been established in vitro that in vivo experiments are required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In the dorsal roots lesion model, we will only make lesions on one side. The behavioural test deficits will be only on the ipsilateral side of the lesion, the normal (contralateral) side will be used as a control to reduce the use of animal numbers. Without stressing the animals and with rationally allocated resting periods multiple behavioural tests will be carried out using the same animal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The dorsal spinal roots and dorsal column lesions will be used during this project. We have been using these models to test transplanted cells to repair damaged CNS axons for more than 20 years. The surgical procedures are less invasive and take a shorter duration compared to others. Animals recover quickly after the surgeries with little effect on their normal cage activities. The functional defects are only shown in the specially designed behavioural tests. The functional tests in this application are based on normal animal behaviours, which are not induced nor forced. The models provide reliable and consistent results and cause the least pain, suffering, and distress to the animals.

Why can’t you use animals that are less sentient?

These investigations constitute an animal model of repair of the human spinal cord and other nerve injuries by transplantation. It is only by studying the method of introducing and incorporating transplanted cells into animals that we can model the human system. Since the criterion of success is the outcome in terms of motor performance and behavioural functions it is not possible to dispense with the animal model.

Rat models are most widely used to study CNS injuries including spinal cord injuries. They have well- understood anatomy and few surgical infections. There are also well-established functional analysis techniques in rats. Although animals such as Zebrafish are less sentient but regenerate their spinal cord after injury, both at larval and adult stages, studies using animals with such spontaneous regenerating ability provide useful information on comparative biology, but they are not suitable for modelling the human condition.

Adult rats around three months are used in this study since the damaged CNS nerves are not able to regenerate spontaneously, which models the human condition.

The therapeutic cells – olfactory ensheathing cells used in this study were originally discovered and obtained in rodents, and the large quantity of research data on their application in neural repair has been from rodent models.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have used these established models with over 20 years of experience. Although we consider there is not a more refined way to conduct these in vivo studies we will, however, regularly review and discuss with the NVS and the UCL representative NC3R's regional manager all the advances in instrumentation and procedures and will integrate them into our procedure if they are applicable. We will continue endeavouring to minimise the invasiveness of the surgical procedures by avoiding any tissue damage, bleeding or infection.

- During the surgical procedure, the level of anaesthesia will be constantly monitored.
- An infection will be prevented by strictly following aseptic procedures and using disposable sterile gloves, dressing packs and sterile instruments.
- Perioperative and postoperative analgesia will be given to reduce pain.
- 5 ml of sterile isotonic saline is injected subcutaneously using a sterile 23G needle at the scruff of the neck region to hydrate the animals.
- After the operation, the animal will be kept warm using a thermostatically controlled heat mat s in a recovery box and supervised until they are fully recovered and moving freely around the cage.
- Cages for post-operative animals have been adapted to have more easily accessible water and areas of increased heating, providing the animal with normal and elevated cage temperature areas. The Diet of post-operative animals is supplemented with wet food e.g. DietGel® 76A (ClearH2O).
- The functional behavioural tests will not start until the animals are fully recovered, at least 1 week after surgery. The tests in this application are based on normal animal behaviours, which are not induced nor forced, to avoid stress on the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

ASPA; PREPARE: guidelines for planning animal research and testing. Laboratory Animals 2018, Vol. 52(2)135–14; LASA Current Publications; ASPA; NC3Rs advice. ARRIVE guidelines 2.0.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will follow closely the advance in research using animals to adopt new animal models with less adverse harm to animals. We will also follow advances in research using tissue

culture, especially with mature tissue to replace animals in the in vivo work and to reduce animal numbers. We will communicate with the Home Office Inspector, NVS, NACWO, NTCO, and NIO to discuss the implementation of potential advanced technology and to periodically view the NC3R's website.

50. Models of Neurological Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

neurodegeneration, neurons, glia, motor system

Animal types	Life stages
Mice	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to investigate the processes and mechanisms of disease in human neurological disease using genetically modified mouse models.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The work is important because there are currently very few effective treatments for neurological disease. This is due in part to an incomplete understanding of disease mechanisms.

What outputs do you think you will see at the end of this project?

At the end of this project we expect to have advanced understanding of neurological disease. Outputs from the project are expected to be scientific publications, but may also include intellectual property relevant to the development of novel therapeutics.

Who or what will benefit from these outputs, and how?

In the short term this research will benefit other scientists and clinicians. In the longer term we hope our research will benefit patients with neurological disease.

How will you look to maximise the outputs of this work?

To maximise outputs we collaborate with other scientists (locally within our institution and overseas) and pharmaceutical companies. We will also disseminate our findings at research conferences and through the media.

Species and numbers of animals expected to be used

- Mice: 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use genetically modified mice as these allow us to work with the most accurate and genetically tractable models that are currently available. The majority of our research currently utilises cultured neurons and glia obtained from embryonic stages, as this allows us to investigate the underlying biology of disease using live fluorescence microscopy. We also use adult mice to characterise disease progression and pathology. For example we are able to investigate specific hallmarks (e.g. pathological, phenotypic) of disease and whether these manifest in genetic mouse models in the same way as they do in human patients.

Typically, what will be done to an animal used in your project?

Typically mice will be born, genotyped, and then used for breeding to generate embryos for experimental studies in vitro. Other mice will be bred and then allowed to reach adult stages to allow us to follow disease progression. Typically these mice will be euthanised in order to obtain tissues for lab-based studies.

What are the expected impacts and/or adverse effects for the animals during your project?

Some mouse models of disease develop moderate phenotypes associated with neurological disease, such as motor symptoms that manifest as reduction in the ability to walk. Typically the models we use also develop pathology in their nervous system associated with phenotypic changes in behaviour.

Genetic models of amyotrophic lateral sclerosis do not gain weight as quickly as their control siblings, but we do not see any signs of adverse effects associated with this.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We anticipate that the majority of mice will show mild severity, with approximately 20% showing moderate severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Ideally we would replace animals with human cell models of disease. However these are not currently available for some of the diseases we work on, e.g. hereditary spastic paraplegia. In addition we sometimes need to study effects that are happening in situ in the nervous system of animals at an advanced age, when clinical symptoms are present. These are extremely hard to replicate in laboratory cell culture systems.

Which non-animal alternatives did you consider for use in this project?

We considered human patient derived cell models.

Why were they not suitable?

In some cases human cell models do not currently exist for the diseases we are investigating. Even if they did, they would not be useful for studying age-related processes involving multiple cell types in an intact nervous system. For example in disorders of the

motor system we need to investigate upper motor neurons, lower motor neurons and muscle. In an animal these are connected by synapses, and this is extremely difficult to reproduce faithfully in cell culture.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This estimate is based on the number of mice we need to maintain in order to preserve breeding colonies of genetically modified animals (100/year) and the number of animals likely to be required for experimental studies (400/year).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For this project we need a regular supply of time mated female mice carrying embryos that will be an appropriate mix of genotypes. Based on experience with these models we require 15 females to be bred using timed-mating each week, in order to ensure that we obtain a minimum of 2 pregnancies. With an average litter size of 7 this give us high confidence that we will obtain at least 2 embryos that are wild type and 2 that are homozygous mutants. These are the minimum required for the live microscopic imaging techniques that we utilise. Fr studies with adult mice we need at least 5 per group in order to obtain reliable results (for example for pathology, protein biochemistry, quantitative PCR).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Over the past 20 years we have reduced the number of animals used for individual experiments considerably. For example we have determined the minimum number of G93A mutant SOD1 mice required for in vivo studies, and optimised breeding strategies to obtain the required numbers of animals for our experiments (e.g. time matings, see above).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using genetically modified mouse models of neurological disease. The majority of our methods use cells and tissues collected from embryonic mice, thus minimising suffering. In a few situations it is necessary to collect tissues/samples from older mice that manifest disease symptoms. In these cases we have optimised husbandry so that the mice experience minimal distress.

Why can’t you use animals that are less sentient?

We are already using embryos for the majority of our work. In some cases we have replaced mice with zebrafish animal models, which are considered to be less sentient. However this is not always possible, in which case we use genetically modified mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Principally we will use animals as early as possible in their disease course, before they develop symptoms classified as severe. In most cases we use embryos, which are essentially asymptomatic.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow NC3Rs best practice guidance, and where relevant we follow specific guidelines for the use of animal models of amyotrophic lateral sclerosis (Ludolph, et al (2010). Guidelines for preclinical animal research in ALS/MND: A consensus meeting. Amyotrophic Lateral Sclerosis: Official Publication of the World Federation of Neurology Research Group on Motor Neuron Diseases, 11(1-2), 38–45.)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To stay informed, we follow NC3Rs on social media to stay up to date with the latest advances in the 3Rs. We also regularly review new publications relevant to our in vivo work. If these advances are applicable to our research we will implement them in a controlled way, to determine whether the measures impact on the number of animals or phenotypes under investigation.

51. Research on Products Used in Prevention and Therapy of Tuberculosis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

BCG, TB, vaccine, safety, potency

Animal types	Life stages
Guinea pigs	adult
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to provide quality assurance that vaccine and therapeutic products used in medicine for tuberculosis (TB) are safe and likely to be effective, including situation of co- infection (TB and malaria). This also includes studies of how these medicines work in a living body.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

As current knowledge advances, new medicines like vaccines and therapeutics products are continuously developed. It is important that we have an improved understanding of whether a new vaccine or therapeutic product is likely to be safe, how it works and how its quality will be regulated. This is to provide assurance that vaccine and therapeutic products used in medicine for TB are safe and likely to be effective. This PPL includes protocols for investigating how these medicines protect in a living body with TB infection or TB-malaria co-infection. A high level of assurance is particularly important for vaccines given to healthy people. If public confidence in vaccine safety and effectiveness falls, vaccine uptake and coverage may be reduced and diseases that were previously well controlled by vaccine can re-emerge. The knowledge gained will be used to inform regulatory process in the UK and worldwide.

What outputs do you think you will see at the end of this project?

The scientific data and new information to be generated from this project will be published in peer reviewed scientific journals and used by both local and collaborative scientists to further our understanding of TB infection and disease (including situation of co-infection of TB and malaria), in terms of pathology, prevention and treatment. These data may also be utilised by regulatory authorities if appropriate.

Who or what will benefit from these outputs, and how?

The potential benefits will include:

1. Evaluation in the quality of product candidates and provision of assurances on the safety and potency of existing and novel therapeutic and vaccine products used in medicine.

2. Provision of information if new generation therapies or new formulations/delivery systems for vaccines antigens are likely to be effective.

3. Research and development studies on correlates of protection that will provide effective methods for potency and efficacy assessment of new vaccines, immunotherapy including cell-based therapies, chemotherapies and related complex therapeutic interventions, including Investigational Medicinal Products destined for clinical trials and will benefit the scientific community.

The knowledge gained will be used to inform regulatory process in the UK, in Europe and worldwide. The data from this research project will also be used by both internal and

external collaborative scientists to further our understanding of TB infection and disease. Specific benefits will depend on the vaccine formulation and the scientific questions addressed but examples include: information of humoral and cell mediated immune responses and whether the response is likely to be protective; the effect of adjuvant; the ability of antigens to stimulate cross protective response to antigen variants; or whether a strain used in live vaccine is sufficiently attenuated and so on.

This project will provide relevant and sensitive assays for biological activity/ quantitation that are developed and applied in clinically relevant settings; these benefit patients and enable the regulatory authorities to make informed decisions on public health issues as well as ensuring prudent and effective use of these powerful biological modifiers. The programme of work has provided, and will continue to provide, a theoretical and experience-based framework for determining the safety and efficacy of existing and potential therapeutic agents for the treatment of human disease using biological mediators.

How will you look to maximise the outputs of this work?

Some of our planned studies in this project are part of the collaborative research projects with external collaborators. Scientific data and new information generated will be disseminated by presentations (oral or poster) in scientific meetings or conferences, and subsequently will be published in peer reviewed scientific journals. This in turns will foster more collaborations with scientists in the UK and worldwide from academic bodies and research organisations working in the same scientific field.

Species and numbers of animals expected to be used

- Mice: 1900
- Guinea pigs: 160

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Small laboratory animals including adult mice and guinea pigs will be used in this project. The use of adult guinea pigs for safety and potency evaluation of mycobacterial products is stated in the standardised protocols used by regulatory authorities, such as in European Pharmacopoeia. Adult (at least 6 – 8 weeks of age) mice with a mature immune system are commonly used small laboratory animals. Their use has been the gold standard for the evaluation of vaccine and therapies for TB because of their small size, low husbandry costs, well-defined health and genetic backgrounds. A large array of tools to investigate immune responses are also available.

Typically, what will be done to an animal used in your project?

Typically, injections or other routes of administration of vaccine or therapeutics will be given to small laboratory animals. Protocol 1 - 4 using guinea pigs have 2 - 3 steps without any repeat. Protocols 5 - 8 using mice receiving immunisation can be repeated up to 4 times. For monitoring of immune responses, mice may undergo multiple whole body imaging step and/or blood sampling step throughout the experiment. Typically, the number of repeats of these steps are 4 - 10 times. Protocol 6

- 8 using mice challenged with TB and/or malaria infection will also be used and TB infection can be repeated one more time. The duration of experiments is usually ranged 2 - 5 months depending on the experimental design. It should be noted that studies involving induction of protection against infection by slow growing organisms (such as Mycobacteria) may need to be continued for many months (rarely beyond 6 months) after challenge for the data to be meaningful. For some routes of administration, anaesthesia may be used and the maximum number of anaesthesia in the lifetime of the animals is rarely beyond 20 times. At the end of an experiment, all animals will be killed by a schedule 1 method or mice may undergo terminal anaesthesia with exsanguination.

What are the expected impacts and/or adverse effects for the animals during your project?

The project including 8 protocols consist of procedures of mild up to moderate severity level. The animals may receive substances such as vaccines by standard routes (for example subcutaneous and oral). Following vaccination, the majority of animals are not expected to experience more than mild to moderate adverse effects (for example skin irritation, local swelling as inflammatory reaction at injection sites).

Blood samples may be taken and this does not expect to have adverse effects for the animals that are more than mild and transient. Non-invasive imaging may be carried out under short duration of anaesthesia and no adverse effects are expected.

Mice may be challenged with TB and/or malaria. TB infection (rare in non-high dose groups and more frequently in high dose groups) may result in moderate adverse effects with gradual loss of body weight (>10%) and reduced physical activities. Malaria infection via mosquito bites may result in swelling, redness or itchiness at the site of mosquito bite. Malaria infection with lethal species may lead to symptoms or adverse events such as gradual body weight loss (>10%), paleness and reduced physical activity if untreated. Combination of TB and malaria infection may lead to symptoms or adverse effects as mentioned above, such as gradual body weight loss (>10%), change of food and water consumption, paleness or changes in observed behaviour or activity level, e.g. pronounced inactivity (‘lethargy’) which deviates from the expected norm during the infection period.

Frequent monitoring and supportive husbandry measures are used to reduce the impact of these adverse effects. At the end of all tests, animals are humanely terminated. The impact of these adverse effects is reduced as far as possible by frequent observations of

the animals by experienced staff and the application of recognised humane end points where necessary.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Based on our experience using the same protocols, under this licence approximately 65% of mice and approximately 10% of guinea pigs are expected to experience mild severity due to the timepoints at which the experiments will be concluded (humane endpoints). Approximately 35% of mice are expected to experience moderate severity and approximately 90% of guinea pigs are expected to experience low moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Some protocols used in this project are performed according to methods described in regulatory guidance. Protection and safety of a biological medicine cannot be fully examined without the use of animal procedures, as multiple factors contribute to protective response and the immune system itself is too complex to be modelled in cell culture. Efforts are continuing to develop suitable alternatives to some of the methods that are retained in this project licence.

The detection of biomarkers of protection could in the long-term result in progression towards suitable

in vitro detection systems to replace some of the in vivo work.

Which non-animal alternatives did you consider for use in this project?

In vitro assays based on viability for live vaccine, such as BCG as surrogates for potency. Molecular biology tests to detect residue virulence gene of BCG or other live attenuated mycobacterial products are in development. In some cases, sufficient information on the immune responses together with results from non-invasive whole body imaging to monitor the biodistribution and persistence of live mycobacterial vaccines in Protocol 5 can be obtained without resorting to the use of infection models (Protocol 6 - 8). For example, antibody titres can be measured using in vitro serological assays or in vitro surrogates of

protection (such as bactericidal or opsonophagocytic assays), cell-mediated immunological responses.

Why were they not suitable?

Many of the tests used to evaluate the quality of new vaccines or vaccine formulations do not involve the use of animals. However, these tests are limited to the assessment of the physico-chemical properties of the vaccine products for production consistency monitoring only. The same for in vitro assays based on viability (for live vaccine, such as BCG) as a surrogate for potency may only indicate production consistency. Thus they can be applied for routine batch release purpose to monitor product quality, but of less value for research purposes where the immunological responses will also be investigated.

There are currently no validated alternatives that can provide reliable and sensitive results for detecting the excessive dermal reactivity and/or absence of virulence of BCG vaccine and other live attenuated mycobacterial products.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimates of the number of animals that will be used in the course of this project are based on experience over the course of the current project licence over the last 4 - 5 years and the predicted maximum usage in the coming 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Research to evaluate the potency and safety of new vaccines is generally hypothesis-driven and the interpretation of data therefore depends on the statistical comparison of an experimental group with controls. Controls would include a group of unimmunised animals and may also include other similar vaccines or components for comparison depending on the hypothesis being addressed. Experiments will be powered to use the minimal number of animals required to produce significant data by a biostatistician. The minimal number will depend on the variability of a particular method. The variability of results can be determined either from pilot studies or from systemic review of literature reporting on a similar model. In general, challenge-based potency tests tend to be more variable and therefore require more animals than their serological counterparts. Experiments may be based on existing pharmacopoeial procedures that have been refined involving

considerable statistical input over the years. These procedures may be used for guidance on the number of animals required per dose

(group) and number of groups. Experimental design and the suitability of particular statistical tests for hypothesis-led research will be planned in consultation with expert biostatisticians. The experimental design will take into account previous experience of similar research whenever this is available, and the use of online tool such as NC3R's Experimental Design Assistant. As a result, this process is considered the minimum required to give reliable results of satisfactory precision.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For some protocols used in this project, the number of animal groups and size of each group are based on optimised and validated methods that are described in regulatory guidance. The number of groups and/ or group size of the other protocols are based on previous experience on small pilot studies, or on information from published literature or collaborators gained with the similar procedure and the advice of experienced biostatisticians. Many tests require the use of one or more control groups and where possible, many test samples will be included together in a single experiment to maximise the use of these control groups and therefore minimise the total number of animals used during the project. New whole body imaging method is also introduced for monitoring distribution of biological medicine within a sedated live animal in order to reduce number of animals sacrificed at various time points.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All procedures used to assess the safety of vaccines are documented in specific pharmacopoeial monographs. The protocols involving infection with TB and malaria are designed to reduce signs of disease and severity, such as frequent monitoring and daily body weight check after high dose TB infection. Although lethal species of murine malaria will be used, it is not intended for the infected mice to develop the disease and show persistent clinical symptoms apart from parasitaemia in the blood.

Once parasitaemia (> 3%) is established, infected mice will be treated with oral chemotherapy (voluntary dosing if appropriate) to clear the infection. For immunisation, no Freund adjuvant will be used, and no adjuvant will be used if intravenous route is required to avoid any adverse reactions. Humane end points are determined such that animals can be killed at the earliest stage of suffering from adverse events which are deviated from normal to minimize the impact of infection on animals. Following experimental procedures (such as vaccination and infection as mentioned above) that adverse effects are expected, frequent monitoring by experienced staff, including out-of-hours checks for some procedures ensure that animal welfare is maintained. Standard husbandry practices include animals being housed in caging suitable for the species used, with a range of varied and appropriate environmental enrichment to allow natural behaviours.

Why can’t you use animals that are less sentient?

All procedures used to assess the safety and potency of vaccines are documented in specific pharmacopoeial monographs that stated the use of guinea pigs. This work will also employ mice from information published in literature or as required from other regulatory guidance or documents on the basis of generating relevant biological effect and suitable dose responses. Because rodents are mammals whose genetic make-up closely resembles that of humans and are commonly used for investigation of human diseases. Many of the TB research questions can be replicated in mice and hence, provide scientists with reliable answers. Previous studies have contributed a significant amount of information on the use particular species or end point.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Efforts continue to refine procedures with moderate severity limits. For immunisation, no Freund adjuvant will be used, and no adjuvant will be used if intravenous route is required to avoid any adverse reactions. Any animals with progressing body weight loss exceeding 10% is a good indicator of mice experiencing progressive TB infection even if there is no sign of physical change or impact on the well-being of the animals. Efforts continue to explore ways to refine end point by introducing frequent monitoring by experienced staff, including out-of-hours checks and daily body weight checks after high dose TB challenge to ensure that animal welfare is maintained. Mice with body weight loss

>10% may be given supplementary diet with easy access to aid improvement. Increase body weighing and monitoring at peak of TB infection have also been introduced.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are different practical guidance available from the NC3Rs website – 3Rs resource library, such as blood sampling, mouse handling and evaluating environmental enrichment.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Information on advances in the 3Rs can be obtained from NC3Rs website – 3Rs resource library. Relevant information will also be disseminated by Named Persons e.g. Named Animal Care and Welfare Officer (NACWO) and Named Veterinary Surgeon (NVS), and by proactively keeping up to date via conference attendance, collaborations, own research etc.

52. Novel Vaccine Development for Porcine Reproductive and Respiratory Syndrome Virus

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Porcine reproductive and respiratory syndrome virus, Pig, Immunology, Virulence factors, Vaccines

Animal types	Life stages
Pigs	juvenile
Mice	juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To develop safer and more efficacious vaccines to aid control of porcine reproductive and respiratory syndrome viruses

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Porcine reproductive and respiratory syndrome (PRRS) remains one of the most economically important infectious diseases affecting the global pig industry, with an estimated cost of over €4 billion each year. Vaccination is a key component of PRRS control. However, current vaccines have safety concerns and provide limited protection, which drives the evolution of an ever-expanding diversity of PRRS virus (PRRSV) variants. More effective vaccines are required to better control PRRSV and alleviate the significant animal welfare and economic burden they cause.

What outputs do you think you will see at the end of this project?

New information on the viral components that determine the severity of disease caused by PRRSV will enable the design of new vaccines that can be safely used to protect pigs.

New information on how arteriviruses dysregulate the immune system by studying lactate dehydrogenase elevating virus (LDV) infection in mice, which could inform the development of improved PRRS vaccines.

Proof-of-concept that engineering live attenuated PRRSV to express immunomodulators can improve their potency.

Proof-of-concept that RNA vectors expressing PRRSV glycoproteins can induce virus neutralising antibodies.

Providing vaccine candidates for further development as products.

All results from the project will be published in Open Access scientific journals once intellectual property has been protected.

Who or what will benefit from these outputs, and how?

The scientific community will benefit from the improved knowledge of PRRSV virulence factors, immune evasion by arteriviruses, and the performance of new vaccine approaches. This could lead to the development of safer and more efficacious vaccines that result in enhanced PRRSV control and consequently improved animal welfare and productivity in the pig industry. This would bring benefits to policy makers involved in livestock disease control, the pharmaceutical and veterinary sector, and the general public through improved food security.

How will you look to maximise the outputs of this work?

All outputs from this project will be published in Open Access scientific journals; this will include unsuccessful PRRS vaccine approaches. Outputs of this work will also be disseminated to other stakeholders and the general public through press releases, presentations at meetings/congresses and social media channels.

Species and numbers of animals expected to be used

- Pigs: 93
- Mice: 114

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The only species known to be susceptible to infection with PRRS virus are pigs and wild boar. The pig is therefore the most suitable animal to study PRRSV infection and to evaluate the effectiveness of vaccine approaches. Weaned piglets will be used as they are most reproducibly infected with PRRSV.

LDV, which naturally infects mice, is closely related to PRRSV. We hypothesise that the principal mechanisms that underlie impaired antibody responses are shared between PRRSV and LDV. We can exploit the wealth of tools available in the mouse, including transgenic models, to better dissect and understand these mechanisms. Findings from the LDV-mouse system, will direct studies with PRRSV in pigs.

Typically, what will be done to an animal used in your project?

Typically, pigs used in this project will be immunised by injection of PRRSV vaccine candidate into the muscle. This will typically be conducted once or twice. Blood and nasal swab samples will be taken at intervals to characterise the immune response and to assess shedding of the vaccine. Vaccinated and unvaccinated animals will typically be challenged once by administration of PRRSV into the nose.

Blood samples and nasal swabs will again be taken at intervals to quantify levels of challenge virus and immune responses. This will typically be done twice weekly. Animals will then be culled humanely to assess lung pathology and tissues will be collected to assess PRRSV loads and for further analysis of immune responses. The typical duration of an experiment is 21-56 days.

Typically, mice used in this project will be infected with LDV by injection once into the peritoneal cavity. The typical duration of an experiment is 14 days. At the end of a study, animals will then be culled humanely to assess virus loads and immune responses.

What are the expected impacts and/or adverse effects for the animals during your project?

Mild to moderate clinical signs for a few days' duration may be observed following inoculation with PRRSV. This will most commonly present as an elevated temperature and lethargy. Sneezing, nasal discharge, coughing and lack of appetite may be observed. No

adverse effects are expected following immunisation with live attenuated PRRSV expressing immunomodulators. However, all animals will be clinically monitored both post-vaccination and -challenge. Assessments and interventions as appropriate will be performed at predefined frequencies in the experimental protocol, including euthanasia on welfare grounds if required. The impact of blood sampling, swabbing and inoculation of vaccine or virus will be both mild and transient.

No clinical signs of disease are expected for mice inoculated with LDV and any adverse effects due to procedures are mild and transient

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The maimum expected severity for pigs that are vaccinated and challenged is mild. Unvaccinated control pigs that are also expected to experience a mild severity due to the selection of a low virulence PRRSV as the challenge strain.

To map virulence factors, pigs are expected to experience mild-moderate clinical signs of disease depending on the PRRSV strain used.

It is estimated that 74% of pigs will be in the mild severity category and 26% of pigs in the moderate severity category.

The maximum expected severity for mice that are infected with LDV is mild.

What will happen to animals at the end of this project?

- Killed
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Due to the complex nature of the immune system, it is not currently possible to study immune responses to vaccination/infection and to determine whether they are protective without the use of animals.

It is necessary to use animals to assess the effects of identified virulence factors on PRRSV pathogenicity and immunogenicity.

Which non-animal alternatives did you consider for use in this project?

Cell culture-based systems will be used to generate and characterise vaccine strains, cultivate vaccine and challenge virus strains and to evaluate virus-neutralising antibody characteristics. In vitro cell- based assay systems will first be used to map and study PRRSV virulence factors before confirmatory studies are conducted in pigs. Expression of immunomodulators by recombinant PRRSV, and their properties, will be analysed in vitro prior to evaluating these as vaccines in pigs.

We will explore murine macrophage cell lines and primary macrophages as an in vitro system for propagating and titrating LDV.

Why were they not suitable?

No replacement options are available to replace the whole animal at this time as an entire organism, including the immune system, need to be present.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers to be used have been estimated using data previously collected from similar studies in consultation with a statistician.

Pilot studies using small numbers of animals will be performed for new investigations. Small animal numbers may be used to propagate and titrate LDV.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Statistical analysis of data collected from previous related studies. Samples will be stored in a biobank, and we will maximise collection of samples post-mortem to facilitate further investigations without the requirement for additional animal experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Use of in vitro models to map and study PRRSV virulence factors and to characterise novel PRRSV vaccine strains. Basing study design on recently conducted relevant studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The only species known to be susceptible to infection with PRRS virus are pigs and wild boar. The pig is therefore the most suitable animal to study PRRSV infection and to evaluate the effectiveness of PRRSV vaccine candidates. .

A well characterised live attenuated PRRSV strain will be selected to be engineered to express immunomodulators. These peptides have previously been evaluated as vaccine adjuvants by our collaborators in mouse models with no adverse effects. Vaccinated and unvaccinated control pigs will be challenged with a previously characterised low virulence PRRSV strain. This enables us to assess protection, by reduction in virus infection, without the animals having to suffer clinical disease.

Previously characterised PRRSV strains of known low and moderate virulence will be used to map virulence factors. In vitro cell based assays have been established that have shown to correlate with PRRSV virulence in vivo. The exchange of virulence factors between these strains is not expected to increase the virulence beyond that of either parental strain.

Mice are natural hosts of LDV, a close relative of PRRSV. LDV infection in mice provides a natural virus-host system to provide insights into how arteriviruses modulate the immune system. This could then be explored in the context of PRRSV in pigs and inform PRRS vaccine development.

Characterised strains of LDV will be used that are not expected to cause clinical disease.

Animals will be inoculated with vaccine or challenge virus in the smallest volume commensurate with the aims of the procedure.

Why can’t you use animals that are less sentient?

The only species known to be susceptible to infection with PRRSV are pigs and wild boar.

The only species known to be susceptible to infection with LDV are mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be housed together with bedding and other items of enrichment. Highly trained animal technicians will monitor these animals throughout the day, ensuring they are comfortable and to maximise their welfare status. We have 24/7 CCTV surveillance which can be used to monitor the animals' behaviour over time.

Pre-study meetings involving the NVS, NACWO and animal services staff will be held to discuss any advances in animal care. Meticulous records will be kept of behavioural, physiological, immunological, and virological measures in order to identify predictive markers and refine humane endpoints. All experiments will be followed by a wash-up meeting to discuss all aspects of the study.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Adherence to the ARRIVE guidelines for reporting these studies, as well as reference to the FELASA guidelines for pig health monitoring to help ensure the most robust health assurance for animals used in this study. FELASA guidelines for administration of substances has been used to limit the maximum volumes for each of the routes.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through continued CPD and frequent review of the CAAT (Center for Alternatives to Animal Testing) and NC3Rs websites, I will keep informed about advances in the 3Rs. Included in CPD will be annual attendance at relevant science conferences.

53. Gene Regulation During the Development of Xenopus

Project duration

3 years 6 months

Project purpose

- Basic research

Key words

development, FGF, myogenesis, transcription, capicua

Animal types	Life stages
Xenopus laevis	adult
Xenopus tropicalis	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to understand how cell signalling regulates gene expression in the context of a developing vertebrate embryo.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There are two main reasons to undertake this work: First, how a single cell (the fertilised egg) gives rise to a complex multicellular organism with hundreds of different types of cells all working together is a fascinating biological problem that underpins life itself and is worthy of investigation for the sake of enhancing knowledge. Second, over the past

decades we have learned that the genes that regulate development are often the same genes that go wrong during human disease, therefore a better understanding of these genes will contribute to more applied studies and lead to better therapeutics.

What outputs do you think you will see at the end of this project?

This project will lead to publications in peer-reviewed journals and dissemination of new information at international conferences including the International Xenopus Conference, the spring meeting of the British Society for Developmental Biology, and the Gordon Conference on Myogenesis. Researchers involved in this project also contribute to outreach and public communication of science in formats such as Pint of Science, an annual international science festival that aims to communicate science to the public by bringing scientists to pubs and cafés to share their research and findings.

Who or what will benefit from these outputs, and how?

The main beneficiaries of this project are:

1. Other scientists: In the short term, dissemination of our findings in research talks and papers will inform scientists in the same or related fields to contribute to their ideas and shape their hypotheses for further research into how cell signalling and transcriptional regulation of gene expression results in cell and tissue differentiation.

2. UK and international society: In the longer term, the researchers trained as part of this project will go on to careers in science and train other scientists. These researchers will contribute to the enrichment of knowledge and enhance the highly skilled workforce in the UK and abroad.

3. Human health: In the long term, a better understanding of the genes that regulate vertebrate development will contribute to the understanding of the molecular basis of diseases, including cancer, and underpin clinical advances in human healthcare.

How will you look to maximise the outputs of this work?

Collaboration is an important part of this project and will help maximise its outputs. We are working with a researcher at another establishment who uses human stem cells to test if our findings in Xenopus can be extended to what happens in human cells. We are also working with another Xenopus researcher at another establishment to investigate how our findings on FGF signalling relate to his findings on Wnt signalling; this will allow us to have a more detailed map of the genetic hierarchies and the cross-talk of cell signalling pathways that regulate development.

Species and numbers of animals expected to be used

- Xenopus laevis: 30
- Xenopus tropicalis: 40

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project seeks to understand gene regulation during vertebrate development and the frog species Xenopus laevis and Xenopus tropicalis are excellent models for this purpose. Both species provide hundreds of embryos on demand, and these embryos develop outside the mother with no need for any complex culture media. The embryos are accessible from the first cleavage stages for experimentation by injection or embryological manipulation. The genome sequences of both species are known and there are impressive post-genomic resources available for data-led projects. Xenopus laevis has the advantage of producing larger embryos more suited to experimental grafting and cultured explants.

Xenopus tropicalis is diploid making gene inhibition protocols easier.

Typically, what will be done to an animal used in your project?

The embryos are used for experiments and destroyed prior to free-feeding and thus are not subject to Home Office regulations, however the adults that provide the embryos are. To procure embryos, a subcutaneous injection of human chorionic gonadotropin (HCG) is delivered to a female frog. This accelerates the natural process of ovulation. Frogs are induced to lay eggs no more frequently than once every 3 - 4 months, in order to allow the re-growth of follicles. Adults are used for no more than 14 spawnings, with at least 3 months rest in between.

What are the expected impacts and/or adverse effects for the animals during your project?

Normally, induction of ovulation by HCG injection has no ill-effects.

Occasionally, the female will suffer from post-induction egg retention which results in death from a swelling of the abdomen. If this happens, it happens within a week after induction and can be monitored by daily inspection for any visible swelling or bloating. When it occurs the animal is culled.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The subcutaneous injection of HCG is a mild procedure, inducing the natural process of ovulation.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In order to study how developmental biology works we need to have access to embryos. Frogs provide large broods of externally developing embryos. These animals are vertebrates: they have backbones, like humans do. This means studying embryos from these animals (rather than flies or worms) is best for understanding many aspects of human development. Frog embryos are excellent model organisms to study developmental processes that happen at the earliest stages of development, because these non-mammalian vertebrate embryos are accessible, developing outside the mother. Gene targeting and over expression methods are very effective in these models, allowing discriminating and informative experiments to generate new knowledge about the molecules being investigated.

Which non-animal alternatives did you consider for use in this project?

Some people study some aspects of development using stem cells that are cultured in 3D to mimic an embryo. These are called gastruloids, embryoids, or organoids. Some aspects of our project can be addressed in this way and we collaborate with another researcher to do this

Why were they not suitable?

The complexity of embryonic development cannot be modelled entirely by explants or stem cell cultures. Within 24 hours a frog embryo goes from an egg to an embryo with most of the body plan organised and most cell types established. This cannot be reconstituted in a dish of stem cells or as embryoids.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Female frogs of both species can be induced to lay eggs up to once every 3-4 months.

To generate sufficient X.laevis embryos for experiments, we induce 2 X.laevis per week during about 30 weeks of the year. 2 X.laevis x 30 weeks per year = 60 inductions per year. 20 X.laevis frogs are sufficient for 60 procedures because they can be induced 3-4 times a year. We estimate 30 animals in total over the project to allow for any death and replacement.

To generate sufficient X.tropicalis embryos for experiments, we use 4 Xenopus tropicalis per week for about 30 weeks of the year = 120 inductions/year. 40 X.tropicalis are sufficient for 120 inductions because we can use them 3- 4 times per year.

These estimates are based on the number of researchers using the animals and the amount of time they spend on animal experiments over the course of each year. Embryos produced from one spawning can support more than one researcher's experiments. The number of animals estimated allows for any death and procurement of new frogs.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

As a group, researchers in my lab coordinate when they do experiments so that when a frog provides a spawning of embryos lots of experiments can be done by different researchers using the same spawning. The embryos are not limiting, so statistically significant results can be obtained from a single spawning. Generating 3 biological repeats requires using eggs from 3 females, so somtimes 3 separate ovulations are needed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Only frogs with a good record of providing high quality embryos are kept in our colonies. The optimal dose of HCG has been determined. The Xenopus community has an excellent platform (xenbase.org) to share best practice across the community using frogs as a model system.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Subcutaneous injection of hormone into female frogs, Xenopus laevis and Xenopus tropicalis.

This is a mild procedure that is carried out by trained staff with a personal licence from the Home Office.

Why can’t you use animals that are less sentient?

Using a non-mammalian vertebrate such as Xenopus to study developmental biology is the most appropriate as frogs produce hundreds of large, accessible embryos allowing many experiments from a single, mild procedure.

What we learn about development in frogs is highly related to other vertebrates including humans, whereas the kind of knowledge gained from studies using invertebrates like flies or worms is less closely related.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have refined our hormone injections into female frogs so that we inject lowest dose to induce ovulation.

We have reduced the density of frogs and fish in each tank which keeps them healthy and productive. We provide enrichment to the tanks, including tunnels and plastic lily pads.

We monitor animals daily for any sign of ill health.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The website Xenbase.org provides a wealth of information on use of Xenopus for the study of developmental biology. Experts from all over the world contribute to the content, which is up to date and trustworthy.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our Facility manager alerts us of advances in 3Rs in emails and as part of our regular user fora.

The website NC3Rs.org.uk is useful for keeping up to date with advances relevant to Xenopus and animal work in general.

54. The Role of Human and Mouse Tissue Stem Cells in Development and Cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Development, Cancer, Stem Cells, Breast, metastasis

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim is to better understand human and mouse tissue and tumour development, the cellular hierarchy of the cells (eg. stem and progenitor) which give rise to more specialised cells, and their interactions with the tissue microenvironment (ie. the environment surrounding the cells). These goals are relevant to human normal tissue development, to cancer progression and cancer spread. An increased understanding may lead to new targeted treatments for the cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In the UK, for example in breast cancer, there are more than 55,000 new cases diagnosed each year and there are greater than 11,500 deaths from the disease. In other major tumour types, the mortality is similar, if not worse. Identification of the regulators of tissue and tumour cell stemness (stem and progenitor cells defined above) versus differentiation is important for understanding cellular signalling that could be targeted to regulate tumour development, progression and spread (metastasis). We believe the proposed pre-clinical experiments based on understanding normal and tumour development and in vitro cancer stem cell (CSC) colony formation and co-culture studies will lead to advances in our understanding of how to tackle cancer progression and its metastatic lethality in patients.

What outputs do you think you will see at the end of this project?

The overall purpose of the current project licence will be to further our understanding of human and mouse tissue development, the cellular hierarchy of stem, progenitor and differentiated cells and their interactions with the tissue microenvironment (ie. the environment surrounding the cells). In order to increase understanding of these biological processes and develop new targets for therapy, we have 3 major outputs:

(i) An increased understanding of the mechanisms and pathways regulating the growth and differentiation processes of normal human and mouse tissues, and their stem cells.

(ii) An evaluation of the role of the microenvironment and pathways such as developmental, cytokines (inflammatory factors) and growth factor signalling in the regulation of human and mouse tumour growth and in particular cancer stem cells (CSCs), their identity, dormant and active states, their regulation and targeting.

(iii) Knowledge of the role of CSCs contributing to the metastatic process (cancer spread to other body organs). The specific questions include: what is the role of the metastatic niche in regulating CSCs and how is the microenvironment altered by stromal and tumour cell signals?

This knowledge, which will be published in high impact peer-reviewed journals, will identify and/or aid development of new therapeutics which will be tested here to target stem cells and metastasis regulators in human cancer prevention and therapy.

Who or what will benefit from these outputs, and how?

The outputs will have a particular benefit for patients with breast cancer, though not exclusively, since this is the main focus of research:

1. We will determine how the tissue microenvironment and specific signalling pathways influence the initiation and development of tumours.

2. We will elucidate how the tissue microenvironment and signalling pathways influence the progression and therapy resistance of cancer.

3. We will demonstrate how the tissue microenvironment and signalling influence the initiation and development of metastases.

We will address these aims experimentally in years 1-5 of the project but dissemination of results and their impact will be fully realised beyond years 1-5 when we would anticipate their publication and their application in clinical trials and other confirmatory and validation studies.

Our data will influence clinical care of patients at high risk of breast cancer, for example those with high breast density (by radio-mammography), lifestyle or genetic factors that increase risk. We anticipate our results will lead to application in cancer clinical trials and other confirmatory and validation studies.

How will you look to maximise the outputs of this work?

Our findings will be communicated to other scientists through collaborations, invited seminars at other institutes and research centres, presentations at scientific conferences and meetings and publication in peer-reviewed journals. The establishment has a policy of ensuring that all publications from scientists are available with free access to all.

Species and numbers of animals expected to be used

- Mice: 1,325

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The work will be conducted in adult mice. These are suited to transplantation of tumour fragments and specific strains can be utilised for transplantation of human tissues without immunological rejection.

Typically, what will be done to an animal used in your project?

For example, this will typically involve the transplantation of human cells into immune-deficient hosts, monitoring growth and cancer spread, testing therapies usually administered by injection or gavage and aimed at interfering in stem cell regulation, tumour growth and overcoming resistance to current therapies. These novel approaches to

therapy may target the tissue or tumour itself or the supporting cells around the tumour implant with the aim of reducing growth or cancer stem cell activity that drives tumour initiation, recurrence and spread to other tissues. Typically, these experiments would be of a duration of several weeks for cell lines and up to 4-6 months for patient-derived tissues or tumour where growth is slow. Procedures might include transplantation of cells or tissue fragments with anaesthesia and pain relief, treatment with an anticancer therapy by mouth or injection, and imaging under recovery anaesthesia to determine growth and spread of the tumour.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of animals (over 95%) are not expected to show signs of adverse effects that impact on their general well-being apart from the development of tumours. The vast majority of the procedures will result in no more than transient discomfort and no lasting harm. However, some mice in which we are investigating the effects of treatments upon the spread and progression of tumours (<3% based on recent experience) might be unexpectedly found dead overnight when monitoring indicates that they are normal. This is due to a sudden impact of the spread of cancer cells from the original location to other body organs, which will be mitigated in future by improved imaging of tumour cells, daily monitoring for shortness of breath and mobility issues. Notwithstanding this, all the mice will be humanely culled at the end point of the experiments.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Based on our experience of using these procedures and experimental models we anticipate about 40- 45% to experience Mild severity, 50-60% Moderate, and less than 1-3% a Severe one (Mild - no significant impairment of normal life; might have injection by needle; Moderate - procedures cause some discomfort and easily detectable disturbance of animals normal state. Loss of body weight, some level of discomfort after surgery; Severe - Animals found dead; Long term disease models where support in feeding is required; abnormal posture). Thus, the vast majority of mice are only expected to experience mildest to moderate clinical symptoms due to tumour growth before they are humanely killed. Some mice will experience the discomfort of repeated (daily) sub-cutaneous or intraperitoneal injections of therapeutic agents or oral delivery for up to 8 weeks. We will aim to utilise the least stressful route of administration wherever possible. However, effective therapies will lead to a reduction in tumour symptoms. There will be some procedures, such as tumour implantation or imaging of tumours that will require short-term procedures under recovery anaesthesia.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Transplantation of human stem cells into a mouse model is currently the gold standard for testing them in an in vivo microenvironment based on experience in the human blood and other tissue stem cell fields. In vitro culture models such as sphere or other colony assays that test stem cell activity exist but these lack the full tissue stem cell microenvironment. Tissue support cells from the microenvironment have important interactions with the stem cells and the developing tissue, the complexity of which cannot be fully modelled outside of an animal. Thus, there is a need for demonstrating stem cell capacity in mice, in order to test the efficacy of factors for stem cell-specific effects in the local environment.

Which non-animal alternatives did you consider for use in this project?

Use of animals will be minimised by using in vitro model systems, for example, colony assays and more complex modelling of tissues ex vivo, eg. using diverse tissue cells grown as artificial organs or whole tissues ex vivo in short term assays. We have published greater than 10 papers outlining ex vivo models and have more in preparation funded by several NC3Rs grants in the past 5 years.

Furthermore, there is a wealth of data analysis that can be carried out on previously generated studies that are publicly available (eg. GEO and c-bioportal) to precede, to guide or to complement in vivo studies.

Why were they not suitable?

These are not fully suitable because whilst progress has been made in the field of cancer research using in vitro models, there are a number of questions that can only be addressed using animal models of disease. For example: variability of disease onset within an animal and within a population (inter- and intra-heterogeneity), investigation of disease progression and therapeutic response, resistances and interactions with the immune system is most faithfully addressed using pre-clinical in vivo models such as mouse. For these reasons, studies on in vivo tumour models need to be performed, in which the benefits are weighted against the likely adverse effects, and humane endpoints utilised.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Number of animals to be used will be determined by the type of experiment and this will depend upon the observations of experiments performed in the lead up to these studies. The overall numbers to be used is based upon numbers used in similar studies on a previous PPL and knowing which studies will be required going forward.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experiments are designed using the principles outlined in the experimental design tool on https://eda.nc3rs.org.uk/ and reported following the ARRIVE guidelines.

Power analyses will be performed to ensure that we use the minimum number of mice to generate significant results. In cases where power calculations are not feasible, group size will be estimated using relevant statistical tests. Moreover, we involve those with statistical expertise, including our bioinformaticians, to ensure that we are using optimum groups sizes, and hence minimum number of mice, in our experiments, we use optimal procedures to reduce the number of mice. For each implantation experiment with a new tumour cell line, a small number of animals may be used in a pilot study to determine their tumorigenic potential and the required number of cells necessary to establish tumours so that the smallest number of animals can be used for the experiment itself.

By the use of stem cell colony and 3D organoid culture studies using normal and cancer cell lines and primary cells, the number of mice needed has been reduced over the course of the previous licence. All of our stem cell studies are started without using mice by using sphere colony assays, 3D organoids, ex vivo tissue fragments and cellular analysis to test effects of regulators or inhibitors on stem cell activity. We will continue this process in this proposed project licence by experiments to mimic tissue environments outside of animals but using the tissue of origin, eg. lung, liver, bone, brain, etc. where normal tissue and tumour cells grow in the body.

For determining the effects of the microenvironment, firstly, small scale pilot experiments will be performed to assess the size of the effect of a particular factor.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot experiments (using up to 4 animals) may be conducted to estimate the biological effect of an intervention (such as a drug) when not known or available in the literature.

We use optimal breeding strategies (conducted on a different licence) to reduce the number of genetically engineered animals.

We take care to ensure that each experiment is appropriately analysed and that the maximum amount of information is gathered thus reducing the need for experiments to be repeated. For example, at the end of each experiment, data is compared to previous studies by appropriate statistical methods (e.g. Kaplan-Meier plots of age at endpoint, Mann Whitney analyses using a two tailed distribution to reduce mouse numbers). We are keen to run experiments in parallel that can share a single control arm, which can sometimes represent a 25% reduction of animals within a given experiment. Where cell transplantation models are no longer needed, cells and / or tissue will be frozen to avoid unnecessary propagation of the model. In vivo experiments are preceded by relevant in vitro experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the appropriate species for transplantation of human normal and malignant cells since immune-deficient mice are able to host the tissues in a mammalian environment that models the human microenvironment in multiple ways.

We will continue to refine experimental protocols wherever possible, to minimize both the number of mice used and their suffering, in particular to refine and reduce the need for surgery by using needles including trocar and transplanting cells in a well tolerated location such as subcutaneously in the flank rather than the ventral region. We will consult statisticians including bioinformaticians in the design of our experiments. Throughout experiments, we will monitor tissue and tumour growth and the health status of transplanted mice with pre-defined frequencies. Any mice that have health related issues related to surgery, transplantation or experimental therapy will be given immediate attention with a view to alleviating symptoms or discomfort and will be killed humanely if adverse effects are considered too severe to be treated.

Why can’t you use animals that are less sentient?

Less sentient animals do not exhibit a similar tissue microenvironment and histopathological features to humans. Mice are far more similar to humans than other suitable and available animal models. This is critical both for using reagents like drugs developed for human targets and for translating findings to the clinic. Cancers develop

over many weeks to months, so use of terminally anaesthetised animals or immature animals is not possible.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal suffering will be minimised by making every effort to keep the tumour models employed at the subclinical levels. Wherever possible, this will be achieved by using non-invasive imaging modalities such as bioluminescence to monitor tumour growth and the development of metastatic disease. In addition, as detailed in the individual protocols, steps will be taken to minimise the severity of the procedures. Finally, we will ensure that all animals receive the highest standard of care, and preventative medicine (including anaesthesia and analgesia where required) will be used according to the general constraints.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Relevant published literature will be used as template for experimental design and decision making (Workman et al., 2010. Guidelines for the welfare and use of animals in cancer research. BJC, 102, 1555-1577).

We will follow guidelines of good practice cited above as well as Morton et al. (Lab Animals, 35(1): 1- 41, 2001). Administration of substances will be undertaken using a combination of volumes, routes and frequencies that themselves will result in no more than transient discomfort and no lasting harm. We will put into practice the guidelines for body condition score (Ullman-Cullere, Lab Anim Sci. 1999 Jun;49(3):319-23) and ageing mice will be monitored and managed according to Wilkinson et al (2020) (Laboratory Animals: 54(3): 225 – 238). We will consult the NC3Rs guidelines and monitor refinement where such practices are published (NC3Rs website and elsewhere).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By reading 3Rs literature, participating in 3Rs workshops locally and nationally, and by our own National Council for the 3Rs (NC3Rs) funding for replacement and reduction of animal studies for our experimental work (we have held 3 NC3Rs grants in the last 3 years and have one shortlisted for funding). Through discussing refinements with our Named Animal Care & Welfare Officers (NACWO), Named Veterinary Surgeon (NVS) and Home Office (HO) inspectorate. We have regular institutional updates such as Annual Project Licence holder meetings.

55. Identification and Validation of Pathology-Related Antigens for Potential use in Immunotherapy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, immunotherapy, new peptides

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our purpose is to identify and to validate new alternative targets of immunotherapies

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Immunotherapy is currently an innovative treatment approach getting more and more relevance in the fight against different immune system related diseases. One example is

cancer, a major cause of mortality in the UK, responsible for over a quarter of all deaths. Even modest improvements in treatment will benefit the lives of thousands.

In the last 5 years, the interest in unconventional peptides has grown rapidly with public and private groups investing resources in this field. In this way these animal experiments are part of a trending project.

This project will be useful to identify and to validate new targets for immunotherapies in mouse models with the aim to move them to the clinic afterwards. A key part of the project is the study of T cells, one type of blood cell of the immune system that play an important role in the immune response. We will focus in the response of these cells against specific peptides (proteins with short length).

What outputs do you think you will see at the end of this project?

The outputs from this work will include:

- Important information about the functional immune system response to new peptides.

- Spontaneous recognition of new peptides by T cells in patients will also provide important information for potential future steps towards translational application of this research programme. Indeed, therapeutic approaches, such as vaccination, could be preferable to Adoptive T cell Therapy (ATT) if we see a significant spontaneous response against these new peptide targets in patients.

- In practical terms, the new information generated will be shared via publications, research presentations at conferences (both national and international), and, if appropriate, the reporting of improved methods.

Who or what will benefit from these outputs, and how?

This work will benefit the basic research community by increasing our knowledge of rearranged peptides (peptide splicing) and the immune system reactivity to this peptides.

We anticipate that outputs of the proposed work could be translatable to cancer benefit and could be commercialised at the end of the research programme. For example, these kind of rearranged peptides could be used for adoptive T cell therapy and vaccination against melanoma, pancreatic adenocarcinoma (PDAC) and colorectal cancer; thus, they will likely be patented.

How will you look to maximise the outputs of this work?

A major mechanism to maximise the output of the work alongside the publication of primary research papers, will be presentation of the work at both big international meetings

and smaller more methods- oriented workshops. These latter formats have the advantage that details of approaches that were ultimately sub-optimal can be shared.

Our group already collaborates widely, and this provides another avenue to share details of approaches that were ultimately sub-optimal and how methods were improved.

Species and numbers of animals expected to be used

- Mice: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The key aims of this work are to identify and to validate pathology specific rearranged peptides as alternative targets of immunotherapies in cancer treatment. This necessitates a mammalian system that contains all the major cell types found in humans, and the mouse is the most appropriate mammalian model. The main cancer types that we study occur in adults, and occasionally, young adults. Therefore, we work with juvenile and adult mice.

Typically, what will be done to an animal used in your project?

Procedures will be performed that lead to the development of cancer in mice. This will be through injected cancer cells or in mice with genetic modifications that lead to tumour development. We will focus on melanoma and KRAS derived tumours (such us, but not limited to pancreatic adenocarcinoma PDAC). With this project we want to test the efficacy of adoptive T cell therapy using T cell clones specific for targets of interest in cancer.

We will use different approaches to identify and validate targets for immunotherapies. In the first case, mice will develop tumours following the injection of tumoral cells. We will treat the animals with modified T cells for tumour specific peptides. The mice will receive this T cells intravenously, and also, other agents intraperitoneally to improve the survival of this cells.

Other approach consists of immunizing mice with a tumour related peptide by injecting the peptide of interest subcutaneously. Then, we will isolate T cell clones specific for that peptide and use the components of that T cells to treat mice genetically modified prone to developed tumours in the same way that the previous model.

Advanced imaging methods (MRI, PET/MR, uCT, SPECT, Ultrasound, Optical Bioluminescence and Fluorescence imaging) will be used to monitor the spread of tumours

and how they react to therapies. Extensive post-mortem tissue analysis will be performed to maximise the information obtained from each animal.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will develop tumours. Initially, these have little effect on the mice, but as they become larger they might affect mobility. Further, animals can lose some weight (up to 15% compared with control age- matched mice). They can suffer a change of behaviour because the less mobility, they could be less active, and in advances stages of disease the can be tachypneic, this is breathing in a higher rate. The tumour could be up to 1.8 cm3, depending on the severity caused by the tumour, the duration of the adverse effect may range from a small number of days to 7-8 weeks (50 days).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate severity is expected for about a 75% of the animals. In the rest of the animals we expect less severity as the treatment of the disease is supposed to decrease the size of the tumours.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are interested in identifying and validating pathology specific new peptides as alternative targets of immunotherapies. Therefore, we need to work with systems where the cells are as similar to the human cells as possible, and also their tissue organisation. The latter point of accurate tissue organisation is particularly hard to recreate using non-animal systems. The mouse is well suited to this as it is a small mammal with relatively simple husbandry requirements. Further, well-established methods exist for genetic alteration in mice, which facilitate further analysis.

Which non-animal alternatives did you consider for use in this project?

Yes, we have considered and use alternatives for much of the work in our group. This includes extensive culture models, analysis of clinical material, and computational

modelling to improve the predictive power of our analyses. Our group has made extensive use of in vitro models (human and mouse) and in silico studies for determining the best peptide candidates and performing a preliminary screening of the efficacy of therapies using these candidates. Also, our group is planning to start the use of human and mouse organoids for melanoma and KRAS derived tumours. The organoids will be expanded from the original tumour and used for the identification of antigens presented on MHC/HLA class I. Through T cell co-culture we will investigate which antigen can be recognized by T cells. In the end, TCRs able to recognize presented antigens will be cloned in T cells and we will test the cytotoxicity against the organoids or different cell lines.

The use of organoids would also allow us to address the issue of the tumour microenvironment, but they can not replace the use of live animals because the microenvironment is not the same compared with a whole organism and the co-culture systems with other cell types are not firmly established. On the other hand, the use of organoids presents different benefits, such us they can be human derived representing human physiology, easier genetic manipulation, they can be specific from individuals.

Why were they not suitable?

It is currently not possible to replicate the complexity of mammalian tissue structures in culture models. A second issue is that the immune system only functions effectively in an organismal context with appropriate white blood cell movement and function within lymph nodes. Finally, we need a whole organism to test therapy failure or success. This is not achievable in simplistic non-animal models due to the differences in the immunosystem.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimate is based on several factors. First, we have taken into account the numbers of mice used in the previous experience. Second, we have taken into account the current number of researchers within the group. Third, we continually re-evaluate the numbers of mice required for each experiment using power calculations. This will allow us to determine the number of animals required per

experiment. By combining this with the group size and availability or resources for data analysis, we are able to estimate how many experiments we will run per year and therefore the numbers of mice required.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experimental design will employ suitable power calculations if prior data exists around the mean and the standard deviation of the metric in vivo. Ideally, experiments will be designed with α (level of significance) = 0.05 and power = 80. If the necessary in vivo data does not exist, then experimental design will be informed by a combination of prior in vitro data generated in the laboratory, existing publications, and the cumulative experience of years mouse tumour work. Experimental Design Assistant (NC3Rs) and PREPARE (Norecopa) will be used to assure best practice. And the experimental groups will be established in a way we can reduce the mice used as controls, for example, the same untreated animals could be use as control for different treatments.

We will perform systematic literature reviews to keep us up to date in the field and minimise the animals needed if data is already published.

On the other hand, regarding the breeding of genetically altered animals, the colony management team at the establishment reviews and manages the breeding of all colonies to minimise excess breeding and we also have tissue sharing to reduce wastage.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We always aim to maximise the amount of data we get from each mouse, for example getting DNA, RNA, and freezing all the tissues for further analysis. Pilot studies will be done to establish the experimental conditions, for example to determine the amount of injected cells.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice in this project. The main focus is to identify and to validate pathology specific new peptides as alternative targets of immunotherapies. In the case of the use of immunotherapies in cancer treatment we will employ sub-cutaneous injections for the generation of tumours or genetically modified mice prone to develop tumours. Mice will receive therapies intravenously, followed by intraperitoneal administration of different

substances to improve treatment. Tumours will be expected to continue growing in the control group, tumours in experimental groups would be expected to stop growing, grow in a slower way or even decrease in size.

On the other hand, mice will be immunized in order to isolate T cell clones. Mice will be injected, for example, subcutaneously with a peptide able to activate the immune system. Mice will be immunized twice or three times with at least 1-week interval between immunizations. Seven days after the final immunization, the mice will be sacrificed and tissues taken for analysis. The main organs of interest will be spleen and lymph nodes to isolate T cells.

Rodents have been chosen as experimental animals as they have the lowest neurophysiological sensitivity while still having an immune system of comparable complexity to the human and are therefore the most frequently used animals in studies of human pathology/immunopathology.

The protocols used involve least pain, suffering or distress or lasting harm for the animals. None of the intended procedures reach beyond the moderate level of severity. Procedures reaching moderate severity arise from the induction of the disease model (cancer) and are thus inherent to the disease studied. Anaesthesia and analgesia will be administered to minimise discomfort and the animals will be monitored closely during all procedures and assessed regularly for any signs of distress. In all the proposed in vivo models, if animals display signs of distress, advice will be sought from the Named Veterinary Surgeon and, if distress cannot be alleviated, the animals will be humanely euthanized.

Why can’t you use animals that are less sentient?

We are interested in identify and to validate pathology specific new peptides as alternative targets of immunotherapies. Therefore, we need to work in systems where the cells are as similar as the human cells as possible, and also their tissue organisation. The latter point of accurate tissue organisation is particularly hard to recreate using reductionist non animal systems. The mouse is well suited to this as it is a small mammal with relatively simple husbandry requirements. Further, well-established methods exist for genetic alteration in mice, which facilitate further analysis.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We try to minimise any possible adverse effects. Regular monitoring by trained staff is in place and we will use the available enrichment in the animal facility. Where appropriate we will also use suitable analgesia and other measures to minimise contingent harms by e.g. tube handling, single use needles, group housing, welfare assessments, adequate acclimatisation, aseptic procedures.

On the other hand, regarding the breeding of genetically altered animals, the colony management team at the establishment reviews and manages the breeding of all colonies to minimise excess breeding and they also have tissue sharing to reduce wastage.

We will also follow local veterinary advice in any moment on care and pain management to minimise any harms to the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We are aware of NC3Rs guidelines and we will also follow PREPARE, ARRIVE and Workman et al guidelines. We also discuss with colleagues in other research groups new improvements that lead to refinement.

The administration volumes and routes of substances is summarized in the animal experience section of each protocol and follows local advice at the establishment.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay up to date via regular communication with animal facilities and Named Persons at the host establishment, other scientists in the field and regular visits to the following website https://www.nc3rs.org.uk/3rs-resources.

56. Neural Basis of Tactile Behaviour

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

neuroscience, electrophysiology, somatosensation

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim is to investigate how neurons in the sensorimotor system operate during tactile exploratory behaviour.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The project addresses one of the fundamental general goals of neuroscience, which is to understand how neurons work to the point that we can accurately predict their activity during behaviour. This kind of discovery research is important not only for its own sake but also because improvement in our knowledge of how neural circuits work will facilitate the development of new treatments for neurological disorders in the future. Past discoveries in this area have led to the development of brain machine interfaces which are starting to become clinically effective.

What outputs do you think you will see at the end of this project?

New information on sensorimotor function, academic publications on the topic and data from the project to be shared with the scientific community.

Who or what will benefit from these outputs, and how?

The short-term benefit will be advances in our basic knowledge of brain mechanisms of sensorimotor function.

In the longer-term, there is potential for benefits both to human health (see above) and to Artificial Intelligence (machine learning) technology. Improved understanding of how neural circuits work makes it possible to 'reverse engineer' principles of how the brain works to suggest new directions for Artificial Intelligence. For example, past advances in our understanding of the brain chemical dopamine has led to important advances in a type of Artificial Intelligence called Reinforcement Learning.

How will you look to maximise the outputs of this work?

New knowledge will be disseminated primarily through publications in scientific journals and through presentation at scientific conferences. In addition, a relatively new trend is to share the data from scientific research with other scientists to a much greater extent than was done in the past. Data, including computer code, will be shared via websites such as github.com. This brings the potential for other researchers to obtain additional insight from the data and therefore for there to be increased benefit from the experiments.

Species and numbers of animals expected to be used

- Mice: 660

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the best species for this project since their brain is structured in a way that is broadly similar to the brains of other mammals (including humans). They permit the use of the most advanced and refined available research methods, whilst also having relatively low species sensitivity. This project concerns how the mature brain functions and hence focusses on adult mice.

Typically, what will be done to an animal used in your project?

Some mice will be implanted with microelectrode devices using aseptic surgical procedures under anaesthesia. Brain activity will be measured in response to sensory stimulation (eg whisker touch) after which these mice be killed.

Other mice will typically be implanted with microelectrode devices using aseptic surgical procedures under anaesthesia. After recovery, using these devices, brain activity will be measured whilst the mice freely explore their environment. In some cases, under anaesthesia, mice will: be injected with plasmids (small pieces of DNA) under anaesthesia, have their whiskers partially clipped, undergo sensory stimulation (eg whisker touch), have brain activity measured in response to sensory stimulation (eg whisker touch) or have minor repair of implants. Once the measurements are complete, these mice will be killed.

What are the expected impacts and/or adverse effects for the animals during your project?

Possible adverse effects are: blood loss (avoided by choosing implantation sites away from major blood vessels); death under anaesthesia (controlled by monitoring of vital signs); post-operative pain (controlled by administration of analgesics and regular behavioural assessment); post-operative infection (controlled by aseptic surgical technique and treatment as advised by a vet).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Expected severity is moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The only way to address the aim of this project is to use animals. This is because the project requires the activity of neurons to be measured with cellular resolution in behaving animals.

Which non-animal alternatives did you consider for use in this project?

in silico modelling

Why were they not suitable?

We use in silico modelling in our research to derive hypotheses of how the brain operates, and such modelling informs the work in this project. However, the data we seek from the current project itself cannot be obtained from purely in silico modelling. This would require detailed knowledge of neuronal anatomy and cellular physiology that is far beyond the state of where neuroscience is today.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers have been peer-reviewed in a successful application for research funding and also have been reviewed by a statistician.

The numbers here have been estimated statistically in order to be confident that sufficient neural data will be obtained to meet our definition of satisfactory data.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A useful refinement is possible by using the latest generation of high-density microelectrode arrays ("Neuropixels"). The current version of these devices consist of ~1000 recording sites and make it possible to record from 100s of neurons simultaneously from one animal. In this way, the number of animals required in order to obtain data from a given target number of neurons is substantially reduced compared to single microelectrode technology. New versions of Neuropixels are under development (but not yet publically available at the time of writing). Thus, we anticipate further reduction benefits to be achievable within the lifetime of this project

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Use of latest generation of microelectrode arrays, explained above.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice. To achieve the aim of the project requires measurement of neuronal activity at cellular resolution in freely moving animals. The way to achieve this that minimises pain/harm is to implant chronic microelectrodes into the brain using aseptic procedures and analgesic drugs, with careful monitoring for the possible adverse consequences, as detailed in the protocols.

Why can’t you use animals that are less sentient?

Mice are the least sentient appropriate mammalian species that are established as a model organism.

Our questions concern how the fully developed brain functions; hence study of animals at immature life stage is not applicable.

Our questions concern the neural basis of behaviour - this cannot be studied under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

An important refinement is that this project moves our research away from procedures requiring restraint and dietry control to increasing use of "free range" procedures where the animals can move freely, where their motivation is intrinsic, and where their dietry intake is ad libitum.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We are using the most advanced microelectrode arrays (see above) and the most recent, refined, published methods for using them.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institute is well-connected with UK 3Rs bodies and keeps scientists here up to date. In addition, the PI is well-integrated in the research community and is highly motivated to use the latest advances (see for example comments on Neuropixels technology above).

57. Myelination in the Central and Peripheral Nervous Systems

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

myelin, neuron, neuroinflammation, neurodegeneration, brain

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project focuses on understanding formation, maintenance and function of the nervous system's myelin and myelinated axons, together forming the nervous system's electrical wiring, under normal and disease conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In the nervous system (brain, spinal cord, optic nerve and peripheral nerves i.e. those nerve connections that lie outside of the brain and spinal cord), nerve cell bodies are separated from each other, often over long distances. The way these spatially separated cells communicate with each other is through a network of ‘electrical wires’ called axons. This communication network is essential for neurological functions such as walking, sight, speech and thought. Axons (the electrical wires) are surrounded by an insulating material called myelin (similar to the plastic coating on an electrical wire) that speeds up the transport of electrically-encoded information along the axons e.g. from one nerve cell body in the brain to another nerve cell body at the base of the spinal cord, or from a nerve cell body in the spinal cord to a muscle in the foot.

The importance of the insulating myelin sheath is highlighted by the fact that humans and animals that cannot make myelin due to genetic mutations, fail to survive beyond infancy. More commonly, diseases that affect myelin result in abnormal myelin being formed (as in diseases termed leukodystrophies) or in myelin breaking down (as in multiple sclerosis). In both these cases, transportation of information (encoded as electrical impulses) along axons is slowed or halted. This usually leads to neurological symptoms such as blurred vision, incontinence, walking difficulties, altered sensation, or difficulty in swallowing. Long-term (chronic) abnormalities in myelin or myelin-forming cells lead eventually to degeneration (breakdown) of axons. Most axons cannot be repaired (as evidenced in spinal cord injury), so this causes permanent neurological disability where the individuals affected become wheelchair- or bed-bound. This happens for example in people with motor neuron disease or multiple sclerosis or leukodystrophy.

A variety of factors cause diseases of myelinated axons including gene mutations (e.g. some forms of motor neuron disease), reduced blood supply (e.g. stroke), physical injury (e.g. from breaking the spine), injury from exposure to toxins or viruses (e.g. polio) and abnormal immune reactions against the body's own cells (e.g. multiple sclerosis). Irrespective of the cause, injury to the myelinated axon is 'sensed' by neighbouring cells that belong to the body's immune system. The role of these cells is to help with repair, but if the injury is severe, these immune system cells can become 'activated'. Long term 'activation' of immune system cells changes them from behaving like 'defenders' to becoming 'attackers', thus causing further damage and speeding up the rate at which disease symptoms progress.

The work described in this project licence is important from a discovery science perspective and a clinical perspective. Only by understanding how nervous system cells develop, function and interact with each other, can we understand what goes wrong in diseases affecting the myelinated axon, such as multiple sclerosis, motor neuron disease and the leukodystrophies. This understanding is essential for treating and preventing such diseases, which each carry enormous financial and emotional costs.

What outputs do you think you will see at the end of this project?

Outputs will be new information that will be disseminated by publication in peer reviewed scientific journals. Outputs will further be disseminated in poster or oral presentation format at scientific conferences; in teaching; to the public at special 'lay person' events; and through social media. New information is expected to include ways to reduce animal use in the study of the myelinated nerve fibre (axon). This is based on a currently funded project from the National Centre for the Replacement, Refinement and Reduction of animals in research (NC3Rs) that aims to develop cell culture models to reduce and/or replace the use of animals to study diseases like multiple sclerosis (MS); a procedure involving induction of MS-like disease in animals, usually mice or rats. A further NC3Rs application is currently being prepared that aims to develop methods to reduce and/or replace the use of animals for imaging cells in the living nervous system, an invasive procedure usually done using mice; the alternative approach will involve using human and mouse cells grown in a dish, for similar purpose.

Who or what will benefit from these outputs, and how?

Members of the laboratory including research trainees, other researchers in the field, collaborators and funders. In the longer term, people with neurodegenerative diseases such as MS, motor neuron disease, periventricular leukomalacia and the leukodystrophies will benefit through improved understanding of the mechanisms of disease, hence through informed approaches to preventative and treatment measures.

How will you look to maximise the outputs of this work?

The applicant has collaborated for many years with colleagues nationally and internationally. Collaborative projects in which we are involved benefit from a wide range of expertise in diverse skills, maximising impact. We publish methods on open science platforms and share methods and approaches with others e.g. we currently provide training to colleagues elsewhere in establishing cell culture models of the nervous system in order to reduce the use of animals in research.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project uses mice with spontaneous (naturally occuring) genetic abnormalities or genetic modifications (where genes have been altered in the fertilised egg from which the mouse line is generated) to understand mechanisms and molecules involved in function, maintenance and disease of the myelinated nerve fibre (axon) such as multiple sclerosis, motor neuron disease and the leukodystrophies. In most cases, mice will be killed humanely for tissue collection or to generate cell cultures. We use mice principally because (i) as vertebrates, their nervous system is similar to ours with respect to myelination (lower species do not form 'compact' myelin i.e. the type of myelin found in the human nervous system), and (ii) because they are available with relevant genetic modifications, such as modifications that enable us to study disease or visualise cells.

Typically, what will be done to an animal used in your project?

Typically, mice will be bred, ear notched (a tiny piece of skin is removed from a specific site on one or other ear) for identification and genotyping (the skin cells are lysed to release the DNA, which is identical to the DNA in every other cell in the body), then killed by a humane method for tissue harvest. Disease models and controls or reporter mice (harbouring tagged cells or organelles) will be killed at postnatal day (P)3, P20, P40, P60, P90, P120, P180 and P365, with a minimum of 3 mice per genotype at each time point; averaging around 100 mice annually. Some disease models and reporter mice (with tagged cells and/or organelles) will be mated and killed at 13 days gestation to harvest mouse embryos. Typically, this will happen to 200 mice, annually. Some mice will receive injection with a substance that labels dividing cells or induces expression of a transgene before being killed for tissue harvest. Typically, this will be one subcutaneous injection and will involve approximately 50 mice annually.

Some disease models will receive a modified diet, designed to reduce the severity of pre-symptomatic disease pathology (harmful changes in cell and tissue that precede the onset of disease signs), and will subsequently be killed by a humane method for tissue harvest. Typically, mice will be killed at postnatal day (P)3, P20, P40, P60, P90, P120, P180 and P365, with a minimum of 3 mice per diet at each time point

What are the expected impacts and/or adverse effects for the animals during your project?

Administration of substances to the body's periphery (usually subcutaneously, i.e. under the skin), such as tamoxifen for gene induction or bromodeoxyuridine (BrdU) to label dividing cells, will be undertaken using a combination of volumes, routes, and frequencies that of themselves will result in no more than transient discomfort and no lasting harm. Local guidelines will be followed for volumes and frequency of dosing when administering such substances.

Some mice with gene defects affecting myelin, axons or both, experience ataxia (loss of full control of body movements) and weight loss after approximately 10 months of age. These disease models will not be maintained beyond one year of age and will be

monitored daily once harmful signs become evident so that the stated severity limit (Level 1 or 2) is not exceeded. Weight loss of 20% or greater or evidence of incontinence or seizure lasting more than one minute or limb paralysis will indicate that the animal should be killed humanely for tissue collection prior to the predetermined end-point.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most animals will be killed humanely prior to the onset of harmful signs (90%). Some will experience mild (10%) or moderate (5-10%) severity harms, being ataxia, whole body tremor, <20% weight loss, seizure lasting less that 1 minute.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We aim to understand aspects of the human nervous system in health and disease, most particularly with respect to myelinated axons (the nervous system's electrical wires). Compact myelin is a specialisation of vertebrates (the term 'compact' refers to the tight wrapping of multple layers of cell membrane around the axon), therefore it is necessary to address scientific questions in vertebrate models, in which the gut, endocrine and immune systems impact the functions of brain cells.

Which non-animal alternatives did you consider for use in this project?

Whenever possible, we will employ partial replacement by using mouse-derived cell cultures, as in current and previous work funded by the NC3Rs. These will be generated from embryonic day 13 mice (occasionally from neonatal mice), meaning that pregnant mice will be used only for harvesting embryos i.e. they will not be used in other procedures. In some cases we will harvest tissue from postnatal mice that have received a dietary intervention aimed at ameliorating (i) pre-symptomatic pathology (cell or tissue injury) and/or (ii) mild or moderate disease signs.

We will review and incorporate alternatives throughout the project as they become available and/or if considered appropriate. For example, we aim to use human induced

pluri-potent stem cells (hi-PSCs) during this project and have established a collaboration for this purpose.

We aim to incorporate mathematical modelling into our work to explore how myelin wraps around the axon.

Why were they not suitable?

Mouse and/or human cell cultures cannot be used for all aspects of the project because they lack some physiological relevance that can only be provided by studying CNS cells in the context of the entire body including the blood-brain barrier and the gut, endocrine and immune systems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is based on previous experience in breeding mutant and transgenic animals for similar work.

Our work uses compound mutants/transgenics, each of which is hemizygous/heterozygous or homozygous for at least two transgenes/mutations, meaning that only a proportion of offspring, ranging from one quarter to one eighth, will harbour the desired genotype combinations.

Many of the mice bred will be provide embryos or pups for cell cultures; others will be used for tissue harvest at a variety of ages; others will receive dietary intervention (aimed at reducing disease severity) and will subsequently be used for tissue harvest at a variety of timepoints post-treatment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The NC3R's Experimental Design Assistant has been and/or will be used to help design robust experiments most likely to yield reliable and reproducible results, including group and sample size based on power calculations performed following collection of preliminary data.

Experimental design, choice of experimental groups, minimising confounding factors, careful handling and ensuring experimental rigour all impact on reproducibility and hence, on numbers of animals used.

Experimental design:

Experiments will be designed to obtain the maximum amount of information from the minimum number of animals e.g. by increasing the size of the control group, the size of the experimental group can be kept to a minimum.

Multiple tissues/samples will be collected from each experimental animal whenever it is possible and appropriate to do so.

Predetermined experimental endpoints (e.g. specific ages) will be adhered to, reducing variability between samples, except if an animal reaches a humane endpoint prior to that time.

Animals of both genders will be used except if there is a scientific reason to use only one gender. Breeding strategies will maximise the chance of obtaining relevant genotypes.

At the earliest stage possible, the experimenter and facilities staff will be blinded to the genotype/manipulation to avoid biased results.

Animals will be randomly chosen and assigned to experimental groups using a suitable randomisation method such as post-selection group assignation meaning that after an animal is selected, the treatment/intervention will be assigned from the random selection of two or more options.

By examining phenotypes across at least three-time points (ages), we will increase the confidence in our findings while keeping group sizes relatively small.

When looking at a new mutation, we will allow a small number of animals to age (usually to 12 months) before analysis, unless a humane endpoint is reached before that. This increases the chance of observing a phenotype if one exists and is much more efficient than examining large numbers of young animals where effects might be subtle or ambiguous.

Experimental groups:

These are usually wild type versus mutant/transgenic. Whenever the genotype allows, we will compare littermates to reduce inter-experimental variability.

Minimising confounding factors:

Where possible, siblings will be housed together and compared to minimise variation between experimental groups due to genetic and environmental factors.

To minimise stress, which can act as a confounding factor in studies of the nervous system, animals will be handled non-aversively (i.e. gently and carefully).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Multiple tissue types e.g. brain, spinal cord, bone marrow and spleen are routinely collected from individual animals and stored for future use if not required immediately. Non-nervous system tissue is routinely shared with other research groups investigating different cell/tissue types.

Efficient breeding practices (e.g. breeding heterozygous/hemizygous mice to generate control and knockout littermates) will be used to decrease variability between comparators and reduce numbers required to obtain statistically meaningful data.

Efficient breeding also includes replacing breeders before reproductive performance declines and replacing non-productive breeders as soon as possible.

Genotyping is carried out early so only suitable animals are maintained and bred; proven stud males will be reused to maximise breeding efficiency (keeping in mind genetic integrity).

Wild type and disease models on the same background strain will be housed together, where possible, prior to endpoint to minimise differences related to the housing environment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use wild type and transgenic/mutant mice to model health and disease in the human CNS. Experimental animals will be handled in a non-averse manner, according to local guidelines, which states "....all laboratory rodents should be handled using non-aversive methods...in all situations where a rodent has to be captured and removed from the cage. "

Specifically, we will use (i) mice with cells and organelles that are labelled with fluorescent markers (coloured tags that can be seen using a special microscope) to allow us to visualise and monitor these cells and organelles (ii) mice producing a protein that switches on genes in a cell- and/or time-specific manner (iii) mice with modifications in disease-relevant genes to study normal cellular processes, disease pathogenesis (mechanisms) and therapeutic approaches, particularly with respect to myelination and glial cell-mediated support of nerve cells.

In most cases, pain, suffering, distress or lasting harm will be avoided because most of the genetic modifications cause none of these. However, some gene modifications cause mild or moderate neurological signs in animals, usually aged 10 months or over. Mice with a harmful phenotype (e.g. signs of disease) will not be allowed to suffer. Such animals will be killed by a humane method when a

*humane endpoint is reached i.e. whilst neurological symptoms are mild or moderate (e.g. whole body tremor, mild incoordination, infrequent seizures of less than 1-minute duration).

*Welfare assessment protocols will be used to monitor adverse effects and determine when humane endpoints have been reached.

The only procedure apart from breeding and maintenance, to be applied to living animals is systemic administration of agent for cell labelling purposes or dietary intervention; the latter being designed to ameliorate (reduce) disease. Otherwise, experimental methods will all be applied to tissue or cells harvested from animals that have been humanely killed.

Why can’t you use animals that are less sentient?

We use mice to understand the human nervous system, most especially the myelinated nerve fibre. Only vertebrates produce compact myelin (meaning that myelin is formed by the oligodendrocyte process wrapping in multiple consecutive layers, tightly around the axon) like that in the human nervous system. Furthermore, mice are available with a variety of genetic alterations that allow us to study disease-relevant phenotypes and visualise cells and cell organelles. Mice are the least sentient vertebrate group, apart from Zebrafish, in which the myelinated nerve fibre can be studied in the context of understanding human nervous system. Zebrafish, whilst useful for studying several aspects of the myelinated nerve fibre, do not replicate all aspects of the human myelinated nerve fibre, in which we are interested e.g. they do express a gene that is mutated in one of the human diseases we study.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice will be housed in an enriched environment (e.g. with nesting materials and/or play tunnels and/or burrowing treats), generally in small groups.

Observations and objective measurements will be used to identify endpoints and if and when reached, the animals will be killed humanely and tissue collected.

The applicant has 23 years of experience in the protocols described in the licence and will provide training and oversight of the use of animals.

Immune-compromised mice, that will be used to generate cell cultures to study e.g. viral infection of the CNS, will be maintained in barrier conditions to minimise the risk of infection.

Mice with neurological deficits will be killed by a humane method if they reach a humane endpoint prior to the predetermined endpoint of the study.

The NACWO and NVS will be consulted regularly and kept abreast of our work.

Routine, careful handling by Biological Services' staff minimises stress when animals are handled in preparation for Schedule1 kill/perfusion fixation.

There might be instances where we need to singly-house animals for scientific reasons, for example, when :

-we have stud males for our breeding program and they fight when kept in social groups.

-only a single male or female is available for experimental intervention that requires specific housing conditions such as a specific dietary intervention.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The ARRIVE guidelines are adhered to as indicated in previous publications from my group and routinely required by the peer reviewed journals in which we publish our work.

Percie du Sert et al - The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research (2020). PLOS Biology https://doi.org/10.1371/journal.pbio.3000410

Sharing and archiving of genetically altered mice will be conducted as described in 'NC3Rs news', December 2021. This procedure has been routinely used in my lab for ~20 years.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The Project Licence holder and members of the research team will ensure continued professional development in the 3Rs area through regular attendance at meetings/workshops such the Animals in Science Regulation Unit (ASRU) annual meeting, 3Rs workshops/symposiums held at local or national Research Institutes, and also attendance at local training workshops organised by the NTCO. The NC3Rs webpage

(https://www.nc3rs.org.uk/) is one we visit regularly. We receive regular updates from NC3Rs through their mailing list.

58. Genetic and Chromosome Instability in the Germline

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

germ cells, spermatogenesis, oogenesis, meiosis, gametogenesis

Animal types	Life stages
Mice	embryo, juvenile, neonate, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to investigate how chromosomes are kept stable in developing eggs and sperm, and why eggs from older mothers often pass the wrong number of chromosomes to the next generation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Problems with keeping chromosomes stable during egg and sperm development in humans typically cause infertility, miscarriage and a range of inherited genetic diseases including Down syndrome.

These errors typically happen when developing eggs and sperm go through a specialized cell division known as meiosis that reduces the number of chromosomes in each cell. These errors are particularly common during egg development, and strongly increase with maternal age. Understanding how eggs and sperm normally keep chromosomes stable during their development, and how ageing affects chromosomes in eggs, will help design strategies that might be able to prevent these errors.

What outputs do you think you will see at the end of this project?

This project will primarily generate outputs in the form of peer-reviewed publicly-accessible research publications in scientific journals.

Who or what will benefit from these outputs, and how?

The immediate beneficiaries of this project will be scientific researchers working in this field. These impacts are likely to be continuous throughout the life of the project.

Medium-to-long term impacts beyond the timeframe of this project include better predictions of whether some types of inherited mutations will or will not cause infertility and related disorders, which has potential to impact on patients.

Longer term beneficiaries might potentially extend to wider human populations affected by changes in fertility, miscarriage and inherited aneuploidy associated with maternal ageing.

How will you look to maximise the outputs of this work?

Output visibility will be maximized through open-access publications and publicity through conventional and social media channels. Outputs will also be maximized through presentation of data and results at scientific meetings and conferences in this research field.

Raw and processed data will be made available on public databases to allow other researchers to study and build on this work, and to identify any links that we might not have been able to find ourselves, potentially using approaches or techniques developed subsequently.

Genetically altered lines developed in this project will be made available to other researchers, and genetically altered lines that are likely to be in high demand will be deposited in appropriate repositories (e.g. MRC Harwell hub of the European Mouse Mutant Archive).

Dissemination of new knowledge that has potential to be translatable to human oocytes will be achieved through collaboration with clinical colleagues associated with IVF clinics.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mouse is a good model to study chromosome behaviour in the germline given the large volume of genetic and molecular knowledge in this area, well-defined descriptions of pathways and identification/analyses of genes involved in these pathways in this species, and the availability of reagents such as antibodies that are used to study this process. The ease of genetic manipulation of this species, and the relatively short generation time are also advantageous. Notably, age-dependent changes in chromosome structure and chromososome segregation in oocytes has already been described in this species.

This work will use mice containing genetic alterations in genes that affect genetic and chromosomal stability in developing eggs and sperm. These animals will allow us to uncover and understand how developing eggs and sperm try to keep their chromosomes stable, and which molecules can be used to influence this process. Non-genetically altered animals will also be used as controls, and to assess age-dependent chromosome changes that happen normally in developing germ eggs.

The choice of life-stages used is mainly determined by the way that eggs and sperm develop in animals and life-stage at which specific events in chromosome segregation happen. Most of the work in this project will be done on adult animals where all stages of meiosis are happening in males, and later stages of meiosis are happen in females. Some work may be done at earlier stages of development to access earlier stages in meiosis in females (mid-late gestation embryos), or to access the first synchronous wave of spermatogenesis in males (prepubertal post-partum pups).

Typically, what will be done to an animal used in your project?

Most (>80%) of the animals used in this project will be used in breeding and maintenance procedures to generate mice with the desired genetic modification. Animals will typically be killed using a schedule 1 method, then post-mortem tissue dissected for subsequent analysis by molecular techniques.

Some mice (~20%) will experience interventions prior to killing and tissue collection e.g. intraperitoneal injection with hormones to induce superovulation up to 48 hours prior to ovarian tissue collection.

A small number (~1%) of mice may experience ageing beyond 15 months and up to 24 months to allow effects on age-dependent effects on chromosome segregation in oocytes to be studied. These mice may also experience intraperitoneal injection with hormones to induce superovulation up to 48 hours prior to ovarian tissue collection.

What are the expected impacts and/or adverse effects for the animals during your project?

Eggs and sperm are not needed for the health of an animal, and though the genetic modifications that will be used in this project might result in subfertility or infertility, this typically will not cause distress or suffering.

The majority (>70%) of animals used for this project will experience breeding and maintenance protocols but will not experience adverse effects given the nature of the genetic modification.

Some animals (~10%) will experience mild adverse effects due to the nature of the genetic modification (e.g. mild intra-uterine growth restriction that does not impact on the ability of the animals to feed or drink, or detectably alter their movement or behaviour).

Some animals (~20%) will experience transient mild threshold pain during intraperitoneal injection of hormones to induce superovulation due to the injection itself.

A small number of animals (~1%) may also experience mild adverse effects associated with ageing to 15 - 24 months.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority (>70%) of the mice used in this project are expected to experience sub-threshold levels of severity.

Some mice (~30%) used in this project are expected to experience mild levels of severity (mild phenotypes associated with genetic alterations, intraperitoneal injection of hormones for superovulation, ageing).

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The specialized chromosome segregation events that happen in meiosis in developing eggs and sperm are currently not accurately modeled in cell lines. Non-animal models such as yeast have provided significant insight into how these events happen in those species, but some of the pathways that operate in this process in mammals are mammal-specific (e.g. Prdm9-dependent regulation of recombination site distribution, Tex19.1-dependent regulation of chiasma maintenance).

Human eggs arrest their development for decades and need to maintain their chromosomes during their arrest. Age-dependent changes in chromosomes during that arrest are thought to underlie age- dependent chromosome segregation errors in eggs, but this is not modeled in yeast or in many non- mammalian species such as nematode worms or fruit flies that use stem cell systems to provide more consistent supplies of developing eggs as they age. Therefore animal models are needed to study mechanistic aspects of this process.

Which non-animal alternatives did you consider for use in this project?

Cell-free in vitro models, cell line models, yeast, nematode worms and fruit flies were, and will continue to be, considered during the course of this project. Notably, some of the mechanisms that will be studied in this project build on information obtained from work from some of these alternative systems, but incorporate mammal-specific components that have a role in preventing chromosomal instability in mammalian germlines.

This project is part of a larger programme of work that will incorporate structural protein predictions, biochemical analysis of purified proteins, and clinical data from infertility patients. The animal use within this larger programme will model and validate key findings from in vitro experiments and clinical data.

Why were they not suitable?

Cell line models do not accurately replicate the chromosome segregation events seen in developing germ cells, and age-dependent changes in chromosomes during a mammalian egg's prolonged developmental arrest are not well modeled in non-mammalian species such as nematode worms or fruit flies that use stem cell systems to provide more consistent supplies of developing eggs as they age.

In vitro biochemical models reconstituting protein complexes involved in this process provide some insight but likely still lack key components and are restricted in scope.

We have previously used animal models to test the in vivo relevance of mechanisms predicted from in vitro biochemical models, and have used discrepancies between our in vivo observations and in vitro models to identify missing components and interactions from in vitro models.

Though cell line, non-animal and in vitro models in isolation are not particularly well suited to addressing this biological question, the animal use in this project is part of a larger programme of work incorporating iterative cycles of testing findings from these models in vivo, and using in vivo observations to develop modelling mechanisms that are relevant to the in vivo physiology.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are based on similar projects currently undertaken on an existing project licence that is due to expire

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The majority of the animals used in this project are used in breeding and maintenance protocols to generate experimental animals of the desired sex and genotype.

I have consulted with a statistician to determine appropriate sample sizes and experimental design to associate genotype with phenotypic effects in these cohorts of experimental and control animals. Data from previous phenotypic analyses were used to generate estimates of data distributions and effect sizes to facilitate those sample size calculations.

I have evaluated technical improvements in superovulation (HyperOva) as a potential route to reduce number of animals used during analysis of oocytes.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have been able to use the increased efficiencies associated with CRISPR/Cas9 transgenics to perform some phenotypic analyses on F0 founder animals, significantly reducing the number of animals used in breeding and maintenance protocols. Though this approach is not optimal for all experiments within this project, experiments designed to

analyse cell-autonomous effects on chromosome behaviours in meiosis in male germ cells are well-suited to this approach.

Cell line models, structural predictions, and biochemical assays will be used where possible to design and test specific types of genetic alteration, or to test functionality of fusion proteins, to identify the key genetic alterations to introduce into mice. This will reduce the number of mice used by limiting the genetic alterations studied to those that are likely to be most informative.

Efficient breeding strategies will be used to limit the number of animals used. Genetically altered lines will be cryopreserved to facilitate sharing and distribution of these lines, and to limit breeding and maintenance once all experimental cohorts have been collected.

Multiple phenotypic assays can typically be performed on tissue isolated from each animal, and some material frozen for use in multiple molecular assays over long time scales, reducing the number of animals needed to generate significant mechanistic insight.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Most of the animals used in this project (>70%) will experience breeding and maintenance protocols, but will not experience any additional procedural intervention, and will therefore experience minimal distress or harm. The genetic alterations typically involve genes and pathways involved in genetic and chromosomal behaviour in the germline - though these can cause infertility this does not cause pain, suffering or lasting harm to the animals. The genetically altered tissue that will be obtained from these animals will be obtained post-mortem after killing by a schedule 1 method.

Some animals will experience additional procedural interventions (e.g. hormone injections to induce superovulation) prior to killing by a schedule 1 method and tissue collection. This is expected to cause mild and transient discomfort, but is not expected to cause lasting harm to the animals.

Superovulations are used to synchronise the oestrus cycle in female mice and allow more oocytes to be collected from each mouse, improving the quality and quantity of research data collected from each mouse.

Why can’t you use animals that are less sentient?

The life stages used for these experiments are largely dictated by the way that eggs and sperm develop: experiments involving analysis of chromosomes during meiosis in males and in later stages of meiosis in females must be carried out on post-natal animals as this is when meiosis occurs during spermatogenesis; analysis of chromosomes in early meiosis in females must be carried out in mid-late gestation embryos as this is when these events occur during oogenesis.

Animals that are less sentient (e.g. nematode worms, fruit flies) do not use the same developmental strategy for oogenesis as humans/mammals, and do not use some of the mechanisms, pathways and molecules under investigation in this project.

Most of the animals used in this project (>70%) will experience breeding and maintenance protocols, but will not experience any additional procedural intervention. The genetically altered tissue that will be obtained from these animals will be obtained post-mortem after killing by a schedule 1 method, therefore terminal anaesthesia does not provide significant benefits.

Superovulations can't be performed on terminally anaesthetised animals given the amount of time that needs to pass between the hormone injections and the tissue collections.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The majority of the animals (>70%) will only experience breeding and maintenance protocols. Refinements associated with this protocol include environmental enrichment, and implementation of non-aversive mouse handling.

Intraperitoneal injections (e.g. during superovulation) will be refined by implementation of non-aversive mouse handling.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs - Responsibility in the use of animals in bioscience research https://nc3rs.org.uk/3rs-resources/responsibility-use-animals-bioscience-research Medical Research Council - Guidance on research proposals involving animal use https://www.ukri.org/councils/mrc/guidance-for-applicants/proposals-involving-animal-use/

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Annual PIL refresher courses run by the establishment will help keep me, and other PIL holders implementing this project, informed about advances in 3Rs.

Experimental interventions (e.g. superovulation) will be authorised locally by the Named Veterinary Surgeon at the establishment through a time-limited Experimental Request Form (maximum duration 1 year), which will allow timely implementation of 3R advances in these regulated procedures.

59. Transgenic Rat Somatic and Germ Cell Gene Mutation Assay

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Transgenic, Risk assessment, Reproductive cells, Mutation, Genotoxic

Animal types	Life stages
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to conduct the Transgenic Rodent (TGR) assay to detect gene mutations in rats in accordance with regulatory guidelines. These studies play a key role in determining the actual risk a test chemical poses to human health and the environment, when non-animal data show that the test chemical could cause DNA damage.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Testing chemicals in this assay will assess whether they are considered safe for use in humans and/or exposure to the environment, or whether they can cause a change (mutation) within the genes of the rat and would therefore be classified as genotoxic. Chemicals which cause gene mutations are potentially very dangerous to humans as they could cause for example, genetic disorders, harm to unborn children and serious illnesses such as cancer.

What outputs do you think you will see at the end of this project?

There are two outputs for this project:

The first will be the use of dose-range finding (DRF) studies for the generation of dosing information that will be used to determine appropriate and scientifically relevant dose levels for use in the main TGR gene mutation test. The maximum tolerated dose (MTD) for a test chemical in the rat will be determined. The MTD is defined as the highest dose that will be tolerated without evidence of study- limiting toxicity, relative to the duration of the study period, but not death or evidence of pain, suffering or distress necessitating humane killing.

The second will be evidence of whether a test chemical is able to cause genetic mutations in any tissue of the body and an understanding of whether this could occur in humans. This knowledge is gained by carrying out the TGR gene mutation test in transgenic rats that have had their DNA modified to be able to detect mutations in any tissue.

Who or what will benefit from these outputs, and how?

The conduct of this assay will be at the request of regulatory authorities, or to meet a regulatory requirement, to confirm that a test chemical does not cause gene mutations in any cell type and is safe to be taken by humans (eg, in the case of new medicines, or food additives), or not toxic to the environment (eg, in the case of weed killers, fertilisers and other chemicals).

The TGR gene mutation test will be conducted in a live animal, where the responses seen after 28 days of dosing will be dependent on how the animal processes the test chemical (eg, how organs like the stomach and liver break down the compound, how the individual cells of the body may be damaged and how that damage might be repaired). Therefore, short-term this enables an accurate assessment of whether the test chemical causes mutations in a living animal and whether it could potentially cause the same mutations in humans.

The TGR gene mutation test enables gene mutations to be seen in any cell type of the body allowing for mutations that could cause cancers or other non-cancer diseases to be detected early and prevented. Germ cells (cells involved in reproduction) can also be assessed, so it is also possible to check if there is likely to be a risk of passing a genetic disease onto an unborn child.

Long-term, if a test chemical is found to cause gene mutations, it will not be approved for sale and its development halted. This is to protect humans and the environment from chemicals which are potentially harmful to human health, which may lead to the development of cancer or other genetic diseases, or be highly toxic in ecosystems.

How will you look to maximise the outputs of this work?

As this test will be conducted as a service, the ownership of the data will belong to the Study Sponsor. Data generated will be sent back to the Study Sponsors by way of a formal study report, which they will use as part of their regulatory package of work to be submitted to the regulatory authorities. Regulatory bodies will then make a decision as to whether the test chemical is safe for approval for use.

Any work that may be conducted on non-Sponsor owned test chemicals will be shared with the wider scientific community through publication.

Species and numbers of animals expected to be used

- Rats: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rats have been used historically in safety evaluations and genotoxicity studies and are recommended by regulatory agencies. The wild-type strain used to make the transgenic rats will be used to determine the MTD.

The use of the transgenic rat has been extensively reviewed by regulators and is identified as being appropriate to use when investigating the potential for a test chemical to cause mutations in any tissue of interest.

Regulatory guidelines specify that rats need to be sexually mature adults aged between 8 and 12 weeks old at start the of dosing.

Typically, what will be done to an animal used in your project?

In the DRF phase, small groups of wild-type animals will be treated daily with the test chemical for a number of days (typically 7 days) in order to establish the highest dose to be used in the TGR gene mutation test. The test chemical will be given either orally, or by injection in to the blood stream. Blood samples may also be taken from these animals to measure, for example, the amount of test chemical in the blood. Animals will be killed humanely at the end of the DRF phase.

In the TGR gene mutation test, animals will be treated, using the same route of administration as used in the DRF phase, with the test chemical daily for up to 28 days. Blood samples may also be taken from these animals to measure, for example, the amount of test chemical in the blood. At the end of the study animals will be killed humanely and the required tissues taken for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

It is expected that up to 60% of animals in the DRF phase will experience adverse effects following repeat dosing. These could include changes to breathing (increased or decreased), body weight loss (up to 15%), occasional changes in normal appearance (e.g. fur may stand up, animals may have discharge from the eyes and nose, or animals may hunch up), changes in normal behaviour (e.g. animals may not interact with their cage mates) and moderate pain or discomfort.

Animals may be restrained for the purpose of dosing or blood sampling into/from the tail vein, which may cause momentary distress. Minor pain or discomfort may be felt when the blood is sampled or test chemical injected.

In the main gene mutation test approximately 80% of animals should not experience any adverse effects, but some in the highest dose group may experience, at worst, moderate adverse effects for a short duration as described above. Any adverse effects will be closely monitored and should not exceed a moderate degree of suffering.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- mild: 60%
- moderate: 40%

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is necessary to conduct the TGR gene mutation test, as it allows time for mutations to occur in any cell of the animal, to fully assess a test chemicals potential to cause mutations in the DNA before giving it to humans or exposing it to the environment. At the moment there is no non animal test available that the regulators will accept for this evaluation.

Which non-animal alternatives did you consider for use in this project?

Prior to animal testing, computer programs which analyse the chemical structure of the test chemical are used to assess how the molecule will interact with the DNA. Cell-based tests are also performed to assess the test chemicals potential to cause gene mutations and DNA damage. These tests are very useful and provide a good indicator of the test chemicals potential to cause mutations in the DNA.

Why were they not suitable?

Computer programmes and cell-based tests are not able to fully represent how a chemical is altered in a live animal system in terms of absorption, distribution, metabolism and excretion by the animal's organs and tissues, nor do they assess whether a mutation has the potential to be passed on to children by occurring in the reproductive cells.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals is based on the number of studies expected to be conducted over the 5 year period, the number of animals required in each treatment group and the number of treatment groups required to enable a statistically significant increase in mutant frequency to be detected.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experiments will be designed in accordance with the regulatory test guideline adopted in 2011 and updated in 2013 and 2020. The guideline includes the latest advancements in

technology and ethical practices to ensure the tests are being conducted properly, using the least number of animals. The number of animals per study group has been shown, using statistical methods, to be the minimum number required to detect a doubling in mutant frequency between non-dosed and dosed animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Dose range finding work will be carried out in small groups of animals so that a dose that can be safely administered for 28 days can correctly be selected for the TGR gene mutation test. This will ensure that the risk of having to humanely kill animals over the 28 days, and potentially having to repeat work in extra animals, is kept to a minimum.

Blood sampling to determine blood levels of the test chemical or other genetic markers identifiable in the animal will be carried out in the DRF and TGR gene mutation test animals, where possible, in preference to using additional groups of animals, or running separate studies, for this sole purpose.

Once an adequate positive control tissue bank and historical database have been established, concurrent DNA would be extracted from tissues in the tissue bank, thus replacing the need to dose a separate positive control group and reducing the total number of animals required on each study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The DRF phase will be conducted in the Fischer 344 rat. This animal has been used for over 50 years in toxicology and cancer studies and was the strain used to generate the transgenic rat, which is used in the TGR gene mutation test. Using the transgenic rat means that gene mutations can be assessed in any tissue from this rat. This model allows the cancer risk of a test chemical to be assessed over a much shorter period of time (typically 1 to 2 months) when compared with conducting 1 to 2 year cancer studies in animals, potentially causing less pain and suffering in fewer animals.

Blood micro-sampling to determine blood levels of the test chemical will be done where possible in preference to collecting larger blood volumes from the tail vein, which could cause bruising and pain, or using additional groups of animals for this sole purpose.

Animals will be humanely killed, and all tissues will be removed post-mortem.

Why can’t you use animals that are less sentient?

The life stage of the animals to be used in this test is specified in the regulatory guideline. Animals are required to be sexually mature adult animals that are 8-12 weeks old at start of treatment.

The preferred rodent species for regulatory toxicity testing and use in cancer evaluation studies is the rat. The Transgenic rat gene mutation detection model is therefore considered the least sentient rodent model that will allow for the data obtained to be correlated with other regulatory toxicity studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be allowed a minimum of 7 days to acclimatise to their new environment following transfer. Animals will be housed in groups and provided with environmental enrichment items as agreed with NACWO. Animals will be regularly monitored for health and welfare and routinely handled by fully trained and competent staff.

During the DRF phase and TGR gene mutation test, animals will be carefully monitored to assess for the onset of any potentially adverse clinical signs. Particular attention will be paid to the animals around the expected peak exposure time to the test chemical. Any observations will be closely monitored until they have subsided, or if necessary, animals will be humanely killed if the humane endpoints are reached.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Experiments will be conducted in accordance with The Organisation for Economic Co-operation and Development (OECD) regulatory guideline. In addition, guidelines issued by the Laboratory Animal Science Association (LASA) and guidance from the NC3Rs on best practice in rodent studies will be followed, as appropriate. In addition, the PREPARE and ARRIVE guidelines will be followed, where applicable.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Although the experimental design is highly prescribed in the regulatory guideline and literature, regular reviews of the latest scientific publications in the field and the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs)

website will be conducted in order to identify and refine any procedures or processes which could be improved upon.

60. Genetic and Environmental Impacts on Hearing

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Genetics of deafness, Progressive hearing loss, Cochlear pathology, Diagnostic tools, Therapies for deafness

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aims of this project are to use genetics to identify critical molecules required for normal hearing, determine the pathological mechanisms underlying hearing loss, develop

diagnostic tools for different types of auditory pathology, and investigate potential therapeutic approaches to preventing the progression of hearing loss or reversing it.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Hearing impairment is very common in the population and can begin at any age. Childhood deafness affects one in 1000 children born and with age this number increases until over half of adults in their 70s have a significant hearing loss. Hearing impairment isolates people from society, is often associated with depression and cognitive decline, and is a major predictor of dementia. The only remedies currently available are hearing aids and cochlear implants, but these do not restore normal function. There is a large unmet need for medical approaches to slow down or reverse hearing loss. This project will provide the scientific underpinning for the development of new treatments.

What outputs do you think you will see at the end of this project?

The outputs will include improved knowledge and understanding of the pathological mechanisms that lead to hearing loss, novel ways of diagnosing different types of dysfunction within the inner ear, molecular pathways that are good targets for development of medical treatments for hearing loss, and proof-of-concept that some types of hearing loss may be reversible.

The knowledge will be published in scientific peer-reviewed journals and discussed at scientific and clinical conferences.

Who or what will benefit from these outputs, and how?

The primary beneficiaries will be people with progressive hearing loss, as we build our knowledge of the mechanisms underlying their hearing which will underpin the development of new treatments. This will be a long-term output.

Clinicians will benefit from these outputs because it will allow them to offer improved services to hearing-impaired patients who currently do not benefit from hearing aids.

The research field will grow as a result of this research, allowing synergy and faster progress towards treatments. The short-term outputs of this project will include this increased knowledge and understanding of the pathophysiology of hearing impairment.

How will you look to maximise the outputs of this work?

All outcomes from the planned research will be published, including findings that do not fit our original hypotheses because these may change the way that researchers build their research plans.

We will continue to collaborate with other researchers in the field including those in the UK, USA, Southern Africa and Asia. These collaborations build a common understanding of the mechanisms involved in hearing loss and spark new ideas.

I will continue to engage with hearing-impaired people, often through my close links with the charity Royal National Institute for Deaf People (RNID). These interactions are inspiring and important in keeping the goal of understanding and treatments in focus.

I will also continue to interact with researchers working in industry because we have a lot to learn from each other about how to develop treatments.

Species and numbers of animals expected to be used

- Mice: 37,700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use the mouse because it is a mammal, and to understand the biological basis of human hearing loss we need to study mammals. Other animals have very different structural specialisations to enable good hearing, and so would be of limited use in understanding human deafness.

The ages of mice that we study are determined by the progression of hearing loss in each different mouse mutant. We aim to analyse the earliest age at which any changes in auditory function can be recorded, so that we can find the primary, or earliest, abnormality that leads to progression of hearing loss. For some of the mouse mutants we study this means analysing them from 12 days old onwards while for others with later onset and slower progression we need to follow their hearing loss up to a year old or more.

Typically, what will be done to an animal used in your project?

Most of the mice we study (over 85%) are culled humanely and their tissues taken for analysis. This minimises the harm they may experience. For the remaining mice, some are injected with drugs to modify the progression of hearing loss and others are anaesthetised, their auditory responses to sounds recorded using methods similar to the human newborn baby screen carried out in hospitals, and then the mice are allowed to recover. Surgery

with recovery is used very rarely when it is necessary to achieve the goals of the project, and every care is taken to minimise any pain or distress.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect any adverse effects for the mice beyond mild stress due to being taken out of their home cage and handled or injected. Occasionally certain mouse mutants may show a predisposition to seizures when stressed, and if this happens and the seizure is more than mild and transient (like freezing) then we will immediately cull the mouse to avoid any suffering. Rarely we may need to carry out surgery on a mouse, and in these cases the mouse will be fully anaesthetised and treated with analgesics for pain relief after the operation. When we need to keep mice beyond a year old, we need to watch carefully for signs of frailty, pain, reduced mobility, growth of tumours, skin lesions, and weight loss. Any mice showing such signs will be treated if possible or culled humanely.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most of the mice (over 99%) will experience no more than mild severity.

Fewer than 1% of the mice used may experience moderate severity, including any mice used for surgery with recovery.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There is no other way of investigating the development and function of hearing apart from using a living animal. We investigate humans with hearing impairment to discover candidate genetic mutations that may explain the deafness, but animals carrying the same genetic mutation are needed to confirm the role of these genes and for understanding the ways in which the auditory system fails, leading to deafness.

Hearing loss is often a progressive disorder and using a mouse we can study the early stages of the dysfunction and identify the main part of the ear affected, but this level of intrusion would never be possible in a human. We need to know the primary site of dysfunction in order to select and assess suitable drugs. It is possible to make some recordings in cultures of samples from the inner ear, but these are not helpful in explaining how the system works as a whole and still require breeding of a mouse to provide the sample to study. It is possible to expose these samples to drugs to test their effects, but this does not tell us anything about the ability of the drug to cross into the inner ear – for this a whole living animal is required

Making observations on other impacts of a mutation on other features in an animal can give us important insights into the pathological and molecular mechanisms involved in hearing loss.

Which non-animal alternatives did you consider for use in this project?

We investigate the genomes of humans with hearing impairment to discover candidate mutations that may explain the deafness. However, animals carrying the same gene mutated or same mutation knocked-in are needed to confirm the role of these genes and for understanding the pathological mechanisms leading to deafness.

Human inner ear samples are available through established repositories, but these are donated after death and usually represent the end-stage of a long ongoing pathological process so often do not tell us why the person had hearing impairment earlier in their life. Using human samples we can only look for correlations and cannot carry out the genetic manipulations to determine the cause of deafness as we can in animals.

We make extensive use of data available on the web, such as protein interaction data to build molecular networks. We work with computer modelling, such as modelling the sources of components of the Auditory Brainstem Response (ABR) to localise the possible site of the malfunction, but generally computer modelling is not helpful because we do not have the necessary information to feed in.

With the help of our collaborators, we make recordings from explanted samples of tissues from the inner ear, but as mentioned above, these have limitations in interpreting the impact upon hearing as a whole because the explants are maintained in such an abnormal environment outside of a living organism with blood circulation.

Other research groups are working on organoid development, and cell lines derived from inner ear tissues are available. However, these have not yet been shown to develop mature sensory hair cells and they have the same limitations as cultured explants from inner ears of animals because they are not in the context of a whole living organism with a blood supply.

We continue to monitor alternatives and will use them when they allow us to answer key questions about hearing loss.

Why were they not suitable?

The non-animal alternatives that we use are useful in some respects, especially the details of genome variants in humans associated with deafness, but, as listed above, these alternatives cannot lead to understanding of the pathological processes (cellular, molecular, electrophysiological) underlying hearing loss. To understand how and why people have hearing loss we need to ask specific scientific questions to establish the causes and mechanisms involved, and these questions can only be addressed by carrying out experiments in animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The total number of mice we expect to use is based upon our experience in carrying out these sorts of research projects previously. The number includes approximately 40% that will be used directly in experiments and 60% that are needed to maintain the breeding colonies of mice each carrying different genetic mutations but not used in experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the minimum number of animals to give us a robust and statistically-valid result based on our previous experience of how variable the results are likely to be. For most experiments, we use 6 mice of each genetic type and age or sex. We adjust the number needed if the variability is larger than we expected. We also consider size of the effect. Small effects (minor worsening of hearing) require large numbers of mice, so we focus upon larger effects on hearing to enable clear interpretation. We minimize environmental influences on findings by studying control mice raised in the same litter as the mutant mice. For drug treatments we use control mice that have undergone the same procedure but without the drug. We collect samples at the same time of day, as daily rhythms can influence the results. Whenever possible we test animals before disclosing the genetic status, to avoid any experimenter bias. Minimising variability in the results means we can use fewer mice to get a statistically-sound result. Some measures can be recorded in the same mouse at different ages, reducing the number of animals we use and allowing tracking of progressive hearing loss in the same mouse.

For statistical testing, we consult experts and websites that give guidance to the appropriate test to use. We consult the NC3Rs Experimental Design Assistant for general guidance.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When we start a new project involving a new mouse mutation, we firstly test the hearing of a pilot set of mutants compared with littermate controls at several ages to establish the rate of progression of any hearing loss, which allows us to select the optimum age for further analysis. We initially test mice carrying either one or two copies of the mutant gene as well as mice with only a normal version of the gene to determine whether we need to analyse all three types in further experiments or whether just mice with two copies can be compared with their normal littermates. For each mouse mutant we study, we set up breeding pairs carrying the correct combination of mutations to produce the maximum number of offspring that can be used in experiments to improve efficiency.

For efficient colony management we use guidance from ASRU (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773 553/GAA_Framework_Oct_18.pdf) as well as advice from the NC3Rs (https://www.nc3rs.org.uk/3rs- resources/breeding-and-colony-management). We regularly cryopreserve samples to allow us to remove the colony from the shelf temporarily and we always archive new mutations we generate via the European Mouse Mutant Archive. Details about breeding performance of each mutant colony are maintained through our electronic mouse tracking database, MCMS, which facilitates identification of key features of breeding colonies and highlights any subviable genotypes.

For new projects involving drug delivery or collection of samples for new analyses, we always read previous published reports describing the methods used and talk with other researchers who have used similar drugs to determine the optimum doses and sample collection methods before planning the experiment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use the mouse because the inner ear of mammals has unique structures involved in progressive hearing loss that are not found in other vertebrates like birds or reptiles. The mouse is the most suitable mammal for the genetic manipulations we need to carry out and there are many existing mouse lines available avoiding the need to use additional animals to generate new model systems.

Most of our experiments (over 85%) involve humane culling of the mouse using approved methods to minimise any chance of pain, suffering, distress or lasting harm to the mouse followed by detailed examination of inner ear samples from the mice. Some experiments include testing auditory responses to sounds under full anaesthesia prior to humane culling while the mouse is still anaesthetised.

A small proportion of our experiments involve testing of hearing under anaesthesia followed by recovery of the mouse, or injecting drugs. Mice used for recovery experiments are observed until they recover fully from anaesthesia to ensure there are no signs of distress. Pain relief will be provided as needed. Surgery will be carried out in only a very small number of mice, and will cause minimal pain or distress but we will monitor this and adapt procedures as necessary to reduce potential suffering.

In the case of new mutations introduced into mice, any possibility of suffering is minimised by daily inspection of new mutants and breeding potentially affected mutants only when needed for experiments.

Why can’t you use animals that are less sentient?

In order to study hearing processes that are most relevant to human deafness we need to study a mammal and the mouse is the optimum mammal to use. Non-mammalian vertebrates and other animals have quite different inner ears and mechanisms for hearing so are not suitable in most cases for analysing the range of abnormal functions that can lead to hearing loss in a mammal.

We carry out some of our measurements of auditory function in fully anaesthetised mice that are not allowed to recover. However, we have adapted some of our recording techniques to allow recovery of the mouse which has the advantages that we reduce the total number of mice needed and we can track the progression of hearing loss in an individual mouse by repeated measurements.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We keep all protocols under continual monitoring to minimise the risks of any harm to our mice. In the case of new mutations introduced into mice, any possibility of suffering is minimised by daily inspection of new mutants and breeding potentially affected mutants only when needed for experiments.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The ARRIVE guidelines are useful reminders of the factors to consider, and we are aware of the recent revision of these guidelines as published by Percie du Sert et al in 2020. We also refer to the PREPARE guidelines published by Smith et al in 2018, the guidance on administration of substances published by Morton et al. 2001, and the LASA guidance on aseptic surgery issued in 2010. For efficient colony management we use guidance from ASRU (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773 553/GAA_Framework_Oct_18.pdf) as well as advice from the NC3Rs (https://www.nc3rs.org.uk/3rs- resources/breeding-and-colony-management).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have access to regular lectures and other resources from the NC3Rs. We have useful feedback from the Animal Welfare and Ethical Review Boards (AWERBs), and several people with responsibilities to inform users about welfare considerations such as the Named Vet, Named Animal Care and Welfare Officers, Named Training and Competency Officers, etc. Occasional circulars from the Home Office, highlighted by regular newsletters from the animal care facility, also are useful in keeping up to date with advances in the 3Rs.

If any of the advances we hear about would improve animal welfare while allowing our research to continue, then we will implement these changes.

61. Vitamin B3 Driven Signals and their Impacts upon Steroid Hormones

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

PARP1, Metabolism, NAD+, Molecular biology, Glucocorticoids

Animal types	Life stages
Mice	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to better understand biology by the study of a molecule called Nicotinamide Adenine Dinucleotide (NAD+). NAD+ is supplied to our bodies through vitamin B3 in our food. In our tissues, NAD+ is the activator of important factors, all of these are potentially critical regulators of health and aid normal hormonal function.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Each organ in our body has very specific and different nutritional and hormonal requirements. These conditions can change during periods of health and illness.

When these demands are not met the organs function is impaired and our health can suffer. However, we currently do not understand the exact demands each organ has for the moelcule NAD+. Moreover, we have only a very simple understanding of how NAD+ levels impact our endocrine systems.

Improved appreciation of these interactions will allow us to identify diseases where hormonal response and NAD+ status might be an important clinical feature.

What outputs do you think you will see at the end of this project?

Outputs of this project will include:

- Datasets providing new information on how the factor PARP1 detects and use vitamin based molecules important to our health and how this factors activity impacts our hormones and varies in different tissues (for example muscle, liver, heart) - these will be shared on publicly available online repositories.

- Publications on the topics of cellular biology and mammalian metabolism. These will give new knowledge on nutrient regulated factors that control health and crosstalk with hormonal systems
  - publications will be in open access journals in the fields of biological research.

- Dissemination of information. Presentations to audiences will be on the topics of vitamin B3 metabolism, molecular activity regulated by NAD+ controlled PARP1 and the interplay between this nutrient driven molecule and hormone systems - findings will be disseminated at national and international conferences.

Who or whatwill benefit from these outputs, and how?

This project may generate new knowledge and information that can be used by other researchers studying human health. Also these findings can be utilised by translational researchers to generate new studies aimed at evaluating treatments surrounding diseases such as muscle physiology (medium term (5years)).

Moreover, it can inform healthcare providers in their treatment of metabolic pathologies that currently lack effective strategysuch as type 2 diabetes, and cardiovascular diseases (long term (10years)).

Longer term studies will be a part of a subsequent PPL.

The projects work will be published in peer reviewed open access journals. Preprints of papers will be published on preprint repositories.

How will you look to maximise the outputs of this work?

This work will be carried out as part of ongoing collaborations with other research groups. Data will be disseminated both at national and international conferences. Unsuccessful

approaches will also be published in journals and shared with relevant communities as technical notes.

Species and numbers of animals expected to be used

- Mice: 1000 mice

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The majority of the current understanding surrounding both NAD+ biology and steroid hormones was generated in the mouse. Mouse genetics and genome engineeering is uniquely advanced (as opposed to fish or fly) and there is a wealth of understanding of murine physiology making mice a most suitable mammalian model.

Adult mice of the strains mentioned within this project have been chosen to avoid any developmental issues that can be seen when treating younger mice.

Typically, what will be done to an animal used in your project?

Mice will be bred using natural mating and may have genetic modifications which are not expected to be harmful. They will be given various substances by injection or in food or water which are not in themselves expected to be harmful. Mice will be humanely killed at the end of the experiment

Typically, under this project animals will experience:

- Tissue collection for genotyping (ear clip).
- Administration of a compound to activate genetic construct. Typically, via intraperitoneal injection, altered diet or altered drinking water.
- A diet with altered constituent for example a diet composed of 60% kcal fat (high fat diet). Food withdrawal for upto 16 hours.
- Administration of a compound to modulate metabolism by the intravenous, oral or subcutaneous routes (typically glucocorticoids).
- Metabolic assessment by using the Sable Systems Promethion home-cage system.

What are the expected impacts and/or adverse effects for the animals during your project?

It is anticipated some animals may experience minor transient discomfort during this project, resulting from the administration of substances, food withdrawal and

acclimitisation in the Promethion metabolic assessment home-cage system. It is expected that some animals will experience minor transient discomfort during this project as a result of administration of compounds and fasting. Withdrawal of food may cause hunger for up to 16 hours but it is not expected than animals will lose weight as they will be able to eat following food withdrawl.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

About 80% of mice will undergo combined procedures (examples include, diet of altered energy constituent and glucocorticoid drinking water treatment or a diet with altered content, administration of compound to alter cellular metabolism and metabolic cage assessment or administration of a compound to alter energy metabolism or typically, glucocorticoid treatment and metabolic cage assessment). Individually, these steps are considered mild but as there are multiple steps this protocol will be classified as moderate severity.

The remaining 20% of mice will not experience combined procedures and be classified as mild.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The physiology of a mouse is very similar to human. The mechanisms and hormones controlling tissue metabolism are likewise similar to humans. This makes the mouse an important animal that can help us understand and develop treatments for human diseases.

In addition, studying a mammalian animal allows us to improve knowledge surrounding the impact of disease. This means we can identify how one tissue (e.g. skeletal muscle) influences other tissues (e.g. liver, fat, heart etc...) and the body as a whole.

Which non-animal alternatives did you consider for use in this project?

Laboratory based In vitro experiments using human and mouse cells are used to underpin and expand findings from this study. They are also used to validate the use of ligands and compounds used to alter cellular metabolism (typically, NAD+ precursors and

glucocorticoids), this better informs us prior to our in vivo work. These elements help reduce our reliance on the animal model as well as informing our planning of animal experiments.

Why were they not suitable?

The use of mice in this work is important to help understand how changes in one tissue (usually skeletal muscle) impact the on the whole body system which is not possible in a laboratory based in vitro model.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimates are derived from previous studies undertaken by myself and my previous research group. In addition published works by groups studying the similar areas.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Colony size is determined for the number of animals required to meet their scientific goal and we will calculate the number of breeding animals required using known breeding data. Prospective sample sizes for experiments have been generated in consultation with biostatisticians using appropriate statistical software in combination with the Experimental Design Assistant (NC3Rs) for generation of power calculations. Animal numbers will be recorded and under constant review to prevent excess animals being generated. Genotyping will be carried out rapidly to minimise animal numbers held under this licence.

Experimental design is based upon our extensive experience and performing pilot studies. Using appropriate power calculations, we have determined that between 6-10 animals per experimental group are required for these studies. Prior to each study, we will outline a protocol for each experiment carried out to identify those objectives for the study, description of the experiment and methods, animal numbers and their identifications to be used as well as experimental grouping allocation and expected outcomes. The PPL holder has experience in statistical analysis and use of statistical software to design experiments with sufficient power analysis. All experiments will be blinded where possible to treatment and planned in accordance with the ARRIVE guidelines.

Typically, indirect calorimetry analysis will require n=8 following experimental intervention (post hoc power analysis n=2; cohens d = 1.66) to reach statistical significance. And for

example altering cellular metabolism with the NAD+ precursor nicotinamide riboside will require n=6-9 following experimental intervention (post hoc power analysis n=2; cohens d = 1.68) to reach statistical significance (recent study of skeletal muscle elevation of NAD+ using nicotinamide riboside experiment: Control=100%

±2.10 vs treatment =126%±4.20 Two way ANOVA p<0.05, n=5).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding will be performed on a minimal basis to reduce surplus mice. Both sexes will be used in studies. Consideration may be given to the use of ex-breeders for the use of tissue collection and analysis.

Pilot studies can be performed to assess the efficiency of genetic recombination and optimal route of administration before moving onto a main study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Models used:

This project will use mice of both wild type and genetically modified backgrounds.
Methods:

- Administration of a compound to activate a genetic construct. Activating compounds are well tolerated and commonly used, doses will be inline with existing publications. Food and water intake will be monitored. Pilot studies will be carried out to ensure we reduce and minimise pain suffering or distress.

- Administration of diets composed of altered nutrient content. Cages will be monitored for cleanliness as in particular high fat diet can crumble onto cage floor. Cages will be cleaned or changed with increased regularity as required. Softer bedding will be used to reduce the chance of excessive grooming.

- Withdrawal of food for up to 16hours. Food will be in place after 16 hours allowing animals to feed as required. Animals will have access to water during these 16 hours. This time period is sufficient to achieve the balance of minimising suffering and generating a metabolic state that is required to better understand fuel source utilisation

during fasting periods, in response to PARP1 deletion and glucocorticoid excess. response to PARP1.

- Administration of compounds to alter cellular metabolism. These compounds (typically glucocorticoids and NAD+ precursors) have been used extensively in the scientific literature and will be used at the lowest dose possible to induce the pharmacological effect required in our mouse lines. The compounds used to influence metabolism are well tolerated and have minimal effects on welfare, and in general terms can often promote metabolic health.

- Mice will be housed in a home cage located in a Promethion indirect calorimetry analysis unit for up to 14 days. Whilst in this system mouse movement, food intake and water intake will be monitored. This replicates the homecage environment and thus minimises any distress.

Why can’t you use animals that are less sentient?

Mice are the least sentient species that will allow us to achieve our objectives. Not all steps to minimise suffering are available to this project. For example use of anaesthesia would impact the biochemical turnover of reactions and the metabolic rate of the animal. This would negatively impact the quality and robustness of the experimental datasets. Also, animal use at a different life stage as a mode to further reduce suffering is unachievable as younger mice are weaning (milk has a high vitamin B3 content, this would negatively influence our data) moreover our objectives are unachievable using mice in in utero.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

High welfare standards, good animal husbandry, and environmental enrichment will be employed. Mice will be carefully monitored and if adverse events are observed, steps will be taken to alleviate them including analgesia, or the affected mice will be humanely killed.

The route, volume and frequency of the administration of substances is reviewed and selected in order to ensure the scientific question can be answered with the least impact on the welfare of the animal.

Where a procedure has an unexpected impact on the welfare of an animal the frequency of monitoring clinical signs and bodyweight assessment will be increased.

Mice will be carefully monitored and if adverse events are observed, steps will be taken to alleviate them including analgesia, or the affected mice will be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Prior to experimentation the PREPARE guidelines (PREPARE: guidelines for planning animal reearch and testing) are used to better support the preparation of animal stuides.

The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments), NC3R's and LASA Good Practice Guidelines are also tobe incorporated.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Literature searches will be routinely performed, I will attend appropriate seminars, symposiums and conferences to find out about new technology and new approaches that can be implemented.

The ARRIVE guidelines will be adhered to. I am subscribed to the NC3R's newsletter and also receive updates from my institutions named information officer.

Close interaction will be maintained with NACWO, NTCO and NIO.

62. Targeted Therapies for Inflammatory Diseases

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Inflammatory diseases, Inflammation biology, Drug discovery, Antibody-based therapeutics

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop new medicines to provide better and safer treatments for inflammatory diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Inflammatory diseases include a vast range of conditions and disorders, including Crohn's disease, ulcerative colitis, rheumatoid arthritis (RA) and psoriasis. These are characterized by an unbalanced immune response, with a vast proportion of patients that do not respond to standard treatments or stop responding over time. Unresolved chronic inflammation has also been associated with the development of cancer, and with different stages of tumour progression and metastasis.

The burden of these diseases is not only detrimental for human health but also brings a notable financial burden to individuals, their families, and society. The work performed under this project licence will help to develop novel therapeutics for the treatment of inflammatory conditions and the diseases associated.

What outputs do you think you will see at the end of this project?

The main outputs of this project licence will be the development of novel therapeutics to rebalance immune and inflammatory responses that drive chronic inflammatory diseases.

Our pipeline is focussing on novel approaches in the pursuing of potential treatments for inflammatory diseases, with some new drugs currently being tested in clinical trials, and several in pre-clinical development. The studies performed within this project will help us to validate molecular targets and test different drug formats.

This project will also help us to identify which diseases can be targeted with these drugs and what other therapeutics could be used in combinations to have a stronger anti-inflammatory effect.

Who or what will benefit from these outputs, and how?

Inflammatory diseases can cause pain and reduce quality of life, by disrupting normal activities and affecting social and psychological wellbeing.

None of the current available drug treatment are curative and in many cases the disease continues to progress, leaving some patients without alternative treatment options. In the short term, this project will provide data that will help identify novel targets and therapeutic strategies. In the longer term, the data generated will support the development of novel therapeutics that will benefit patients either as stand- alone therapies or in combination with other agents, increasing response rates and ultimately being able to bring about effective disease control in a greater number of patients. This would benefit not only individual patients, but it will also help to relieve the burden placed on communities, workplaces, and national health systems.

How will you look to maximise the outputs of this work?

Data generated in this project, including any unsuccessful approach, will be disseminated within our organisation and it will benefit several programs of work.

Findings will be presented at national and international scientific conferences and meeting as posters and talks, and eventually published as scientific articles and patents.

The results of the potential accompanying clinical trials will be included in the entries to the ClinicalTrials.gov and/or published in the scientific journals.

Species and numbers of animals expected to be used

- Mice: 5350

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In this project, we are developing antibody-based therapeutics against inflammatory diseases.

Mice are used to model inflammation because of the relative ease of manipulation of the immune system and well understood genetics. We will be using young adult mice (typically 6-14 weeks old at the start of the study), with a fully mature immune system.

Typically, what will be done to an animal used in your project?

In this project, mice will be challenged with a stimulus that will induce an inflammatory response. The administration of the stimulant could be done locally (e.g. in a subcutaneous pouch) or systemically (e.g. via intravenous injection). When using inflammatory bowel disease models, the inflammation will be induced in the gut. Some of these animals will also receive an injection of a colonic carcinogen to induce the formation of inflammation-associated carcinoma.

Mice will be treated with novel anti-inflammatory drugs, mainly antibody-based therapeutics dosed generally 2-3 times a week for up to 3 weeks , but on some occasions, treatments may be administered more often. During the study mice will be observed, weighed and sometimes imaged, usually 2-3 times a week. The studies will be terminated when enough data on the effects of the tested treatments have been collected and no additional scientific knowledge could be obtained within welfare guidelines.

What are the expected impacts and/or adverse effects for the animals during your project?

Induction of local or systemic inflammation may cause mild pain and some behavioural changes, including decreased peer interaction. Body weight loss may also be observed. Mice will be regularly monitored, and symptoms alleviate whenever possible, for example with provision of food supplement on the cage floor.

When using colitis models, mice will experience intestinal inflammation, with expected adverse effects including the presence of blood in the faeces, mild abdominal pain and mild to moderate weight loss.

Most of the animals used in this project will be dosed with antibody-based treatments. Antibodies are usually very well tolerated, but their administration could lead to temporary mild adverse effects such as weight loss or piloerection. These effects usually resolve within 2 days from appearance.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most of the individual procedures performed under protocols 1, 3, and 4 are expected to result only in mild discomfort. However, as studies run under these protocols will include several procedures, this will result in a cumulative level of severity likely to be moderate for most of the animals. We estimate that around 50% of the mice in Protocol 1 and 70-80% of the mice under Protocol 3 and 4 will experience cumulative moderate severity. For the remaining mice used under these protocols we estimate mild severity.

Mice used in Protocol 2 (IBD model) are expected to experience moderate severity due to the induction of colitis (>80%)

Most mice used under Protocol 5 will experience procedures only under terminal anaesthesia, thus they are expected to be classified as non-recovery.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Inflammation is a process that involves many immune and non-immune supportive cells, including macrophages, neutrophils, T-cells, fibroblasts and endothelial cells. All these different cell populations communicate between each-other through complex positive and negative feedback loops of inflammatory mediators. This intricate interaction can only be modelled properly in an animal setting.

Chronic inflammatory conditions have also been associated with the development and progression of malignancies. Studies performed under this licence will allow us to

investigate the anti-inflammatory properties of our novel therapeutics and their ability to counteract the process of inflammation- associated carcinogenesis, process that could not be modelled without the use of animals.

Which non-animal alternatives did you consider for use in this project?

It is possible to model some properties of inflammatory diseases by expanding and stimulating human tissues in vitro or with 3D cell cultures such as spheroids or organoids from human or animal organs.

It is also possible to study inflammatory processes from tissue resections from the patients, for examples from surgical residual material or biopsies. However, these can be very difficult to obtain.

Why were they not suitable?

The current 2D and 3D in vitro culture systems are very simplistic, as they include only few cell types, they can't recapitulate the complex interaction between the several immune cell populations. However, these remain a useful resource and we and others are working towards more refined 3D culture systems.

Patient explants are more relevant models, but they cannot be maintained in culture for more than just a few days. Some aspects of the inflammatory process take a long time to fully develop and therefore cannot be studied in this system. Also, the limited amount of these types of tissue can restrict the number of conditions/treatments that can be tested.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals we will use in this project based on the number of targets and drug candidates we plan to test yearly during this program.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To ensure we use the minimum number of animals, experiments will be carefully designed, taking into account the expected outcomes and statistical analysis of the data. An estimation of the animal being used in this project was done through power and sample size calculation, for example, to compare two means (e.g. control vs treatment), a sample

size of n = 5 mice per group will allow to detect a Cohen'D effect size of 2 (large, acceptable for data with low variability), using a two-sided t-test analysis with a type I error of 5% and a power of 80%. Data previously generated in the literature, together with pilot experiments, will help to have preliminary information that will be used in each individual experimental design. Power calculations will be performed using the NC3R's Experimental Design Assistant, Gpower3.0 or other relevant software.

Sources of variability (cage identity, sex, age, operators etc.) will be controlled. Stratification may be used to guarantee balance between the groups, and animal assignment to experimental groups will typically be randomized. For models with a confirmed sex bias (e.g. sex differences in experimentally induced colitis), only the sex with the desired phenotype will be used. Alternatively, experiments will be performed on comparable numbers of male and female mice whenever possible.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All our antibodies and related molecules undergo extensive characterization of their activities in a suite of cellular and tissue assays in vitro. Only a small number of selected candidate molecules (usually less than 5) is used in animal experimentation.

To further reduce the number of mice used, we will maximise the amount of data obtainable from each single experiment. Whenever possible, for example, we will obtain preliminary information on the levels of the drug in the mouse blood post injection while testing its general tolerability or during pilot studies. This may allow us to identify any dose-related issues with a specific molecule and guide us in the optimisation of the dosing regimens.

We routinely use the micro-sampling technique for blood collection from tail vein for plasma isolation. Using this method, we can collect blood from the same animals over multiple time points and generate data using a reduced number of animals compared to standard blood collection methods. We are also planning to implement live imaging for the longitudinal analysis of the pharmacodynamic activity of our drug, which will help reduce the number of mice needed.

Additionally, collecting mouse tissues and performing ex vivo experiments will allow us to further reduce the number of live animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use commonly used mouse models of inflammation, such as localise inflammation (air pouch model), systemic inflammation model and disease-specific model (inflammatory bowel disease).

These models have been widely used to strengthen our understanding of human immunology and to help define the pathophysiology of immune-induced inflammatory diseases. The large body of literature available on these animal models will help us define the most efficient means of obtaining scientific data while causing the least harm to the animals.

Why can’t you use animals that are less sentient?

In this project, we will investigate therapeutic interventions that modulate the immune system. Many of the targets for these interventions (both particular cell types and proteins) are not present in non- mammalian species. For this reason, mice with their very well understood genetics and immunology, plethora of well-established inflammation models, and developed technologies to manipulate their genome are currently the species of choice for modelling inflammatory diseases.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice will be housed in a state-of-the-art animal unit where a high standard of environmental enrichment is usually provided to animals. Animals will be housed in groups to allow for social interaction. However, when required for animal husbandry purposes, they might be housed singly and enhanced environmental enrichment may be provided.

When appropriate, suitable analgesia and anaesthetic regimes will be used to minimize animals suffering. Where novel agents are administered, the duration and frequency of monitoring will be increased to ensure that no animal will suffer unduly. Humane endpoints will be continuously evaluated for each model and refined wherever feasible.

All studies will be reviewed internally by the Project Licence holder and the personal licence holders involved before commencing to ensure that experiments are aligned with the Licence and reflect the implementation of best practices.

All the compounds used in animal studies will undergo extensive quality control checks and where possible, we will use drugs sourced at clinical grade.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The PREPARE guidelines will be used to assist with the planning of experiments and the LASA Diehl guidelines Guidance on dose level selection for regulatory general toxicology studies for pharmaceuticals. The “Guiding Principles for preparing for and undertaking aseptic surgery” (LASA 2010) guidelines will be followed for surgical procedures.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All staff involved in the design and implementation of animal experiments in line with this project licence will be encouraged to participate in relevant inflammation modelling conferences, courses and workshops to stay on top of current trends in the field.

Additionally, they will register on the NC3R mailing list to ensure communication of best practices. Periodical reviews will occur internally and in consultation with the named people to ensure best practice is implemented at all times.

63. Efficacy Testing of Antiviral Compounds and Vaccines for Emerging RNA Viruses

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

virus, vaccines, antiviral, therapy

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To develop improved vaccines and drugs for the prevention and treatment of disease caused by emerging viruses

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The COVID-19 pandemic has emphasised the need for continued efforts to develop effective antiviral drugs and vaccines against emerging viruses, particularly viruses with an RNA (as opposed to DNA) genome. Effective antiviral drugs are an important first line of defence against an emerging virus until a vaccine is developed. The plasticity of RNA viruses means that they can evolve rapidly to resist inhibition by viral-acting drugs or evade immune responses developed in response to vaccination.

There is therefore an increasing focus on compounds that act against proteins in host cells that are hijacked by viruses to support their replication. These host-acting compounds carry less risk of development of drug resistance and are usually broader acting (i.e. effective against unrelated viruses) than viral-specific drugs. Although effective COVID-19 vaccines were rapidly developed, further improvements to vaccines against RNA viruses such as COVID and influenza would be the generation of immune responses that are not compromised when viral variants arise. Furthermore, a challenge for developing vaccines against respiratory viruses is the induction of immune responses at the respiratory mucosal surfaces to block infection early. Despite the rapidity with which COVID-19 vaccines came into clinical use and decades of vaccine research, there are still some viruses for which safe and effective vaccines are not yet available. In particular, development of vaccines against flaviviruses including dengue and Zika has faced the challenge that it is possible to generate an inadequate immune response that can lead to more severe disease on subsequent exposure to the virus due to a phenomenon referred to as 'antibody-dependent enhancement'.

What outputs do you think you will see at the end of this project?

The project is likely to generate publications in peer-reviewed journals and provide proof-of-concept data to enable additional funding to be secured for the further development of antiviral drugs and vaccines. The expected outputs for objective 1 are data demonstrating the immunogenic potential of novel vaccines. Results will include titration of antibodies in serum samples and measurement of T-cell activation (e.g. using ELISpot assays). Outputs expected for objectives 2 and 3 are demonstration that novel vaccine formulations are effective at protecting against respiratory viruses or antiviral drugs are effective at preventing or treating infection with respiratory virus. This will be demonstrated by a reduction in clinical signs between treated and untreated groups, reduction in virus replication (measured by titrating infectious virus in nasal washes or lungs collected post mortem and reduction in lung damage (detected by histological examination of lung tissue).

The project will provide proof-of-concept data about vaccine formulations and aniviral compounds that will form the foundation for further development towards clinical application in both the human and veterinary medicine fields.

Who or what will benefit from these outputs, and how?

The academic community via publications arising from the studies. This will likely be in the medium to long term.

Individuals (both human and animal) affected by disease caused by emerging RNA viruses may benefit from new antiviral treatments and vaccines in the long term.

How will you look to maximise the outputs of this work?

I have a strong track record in disseminating findings from my research via publication in scientific journals and lay articles and presentations to both scientific and lay audiences. I am engaged with industry and have experience of the requirements for licensing antiviral drugs and vaccines and also collaborate extensively nationally and internationally.

Species and numbers of animals expected to be used

- Mice: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are used because of the long history of antiviral drug and vaccine development in the mouse model. This means that experiments to demonstrate efficacy of antiviral drugs and vaccines have been refined to the greatest extent in mice. Adult mice are used for vaccine immunogenicity testing because a mature immune system is required to generate antibodies and cellular immunity to vaccines. Testing the efficacy of antiviral drugs and vaccines requires that infection of untreated animals exposed to infectious virus produces measurable outputs (such as clinical signs of disease, virus replication) in order that a reduction in these outputs can be observed in treated animals.

Typically, what will be done to an animal used in your project?

The majority of animals will have either an antiviral drug or a vaccine administered by one of several routes. The administration of vaccines or drugs is unlikely to impact adversely on the animals as this would not be acceptable in the final product. The principles for protocols of minimal severity will be followed. For most vaccines, one or two 'booster' immunisations are required, and antiviral compounds may require repeated administration over several days. The level of circulating antibodies in the blood (the antibody titre) in response to vaccination will be monitored by blood sampling at appropriate time points.

The typical experience of animals undergoing a non-recovery protocol for the collection of blood and tissue at the end of experiments would be exposure to an anaesthetic gas or the experience of an injection of an anaesthetic drug. The anaesthetic agents would be administered in such a manner as to minimise the risk of panic and the animals would gradually lose consciousness and enter a stage of deep surgical anaesthesia. At this point blood and/or tissues would be collected. The loss of blood during the procedure would ensure that the animals would be unable to regain consciousness; however humane killing would be completed and confirmed according to Schedule 1 of the Act.

Some of the mice will be experimentally infected with a respiratory virus. This will be done by dripping a small volume of infectious viruses into the nostrils of the mice.

The longest experiments (up to around 8 weeks) will be those in which two or three immunisations of a vaccine are required to generate sufficient immune response followed by inoculation with virus to test the efficacy of the vaccine.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals will experience mild and transient effects when antivirals and vaccines are administered, the extent of which may depend on the route of administration with intramuscular or intravenous administration requiring a needle and syringe. Intradermal administration using a ‘gene gun’ may also cause transient pain; intranasal administration is less painful but may cause some discomfort. Vaccine immunisation may lead to a period of mild lethargy for typically up to 24 hours. The recognised and characterised adverse effects of the adjuvants used to stimulate the immune system may result in localised redness and inflammation for a period of typically 3 to 5 days. In some cases, a subsequent non painful sterile abscess or granuloma will form at the injection site. These may not resolve during the time scale of the immunisation protocol but will be monitored by the Named Veterinary Surgeon.

When animals are blood sampled in a conscious state, use of refined techniques in line with LASA and NC3Rs guidelines will ensure that any pain is mild and transient in nature. On rare occasions (<5%) a bruise or swelling at the site of blood sampling may form, which should typically subside within 3 to 5 days.

Challenge infection with infectious respiratory virus will cause control animals (i.e. animals that did not receive an active vaccine or drug) and possibly also treated/vaccinated animals to experience clinical signs typical for that virus in mice. As the mouse is not a natural host for respiratory viruses affecting humans and/or livestock or companion animals, the clinical signs experienced by mice are typically mild. There are some strains of influenza A virus (e.g. ‘PR8’) that have been adapted to cause more severe disease in mice so that the benefits of vaccination or antiviral treatment are more obvious.

Rather than develop fever, mice can become hypothermic when infected with PR8. They typically

develop anorexia and demonstrate behaviours consistent with physical discomfort or lethargy, such as huddling, hunching, and fur ruffling from lack of grooming and dehydration. Mouse-adapted influenza strains can cause a fatal primary viral pneumonia. Therefore, mice challenged with these strains will be killed 4 or 5 days after infection (before they are severely affected) and virus infection confirmed by analysing the lung (and potentially other tissues such as nasal turbinates) to detect virus or virus damage using histochemistry and titrating infectious virus. For respiratory syncytial virus, clinical illness is only seen in older mice, manifested by ruffled fur, reduced activity, and weight loss.

For animals undergoing blood sampling and/or tissue collection under terminal anaesthetic, the deep surgical anaesthetic plane will prevent animals feeling any pain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity experienced by animals during administration of vaccines or antivirals while conscious will be mild. This will apply to all animals on such protocols.

The expected severity experienced by animals undergoing blood sampling while conscious will be mild. This will apply to all animals on such protocols.

The expected severity experienced by animals after virus infection will be moderate. The actual severity experienced by individual animals will depend on on the virus strain used and dose, and whether animals are in non-treatment groups and/or the efficacy of the experimental vaccines or treatments.

The expected severity of this work is non recovery for all animals from which blood is sampled under terminal anaesthetic.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Efficacy testing of vaccines and antivirals intended for use in humans or veterinary species involves the whole complexity of the immune system of higher order animals.

Which non-animal alternatives did you consider for use in this project?

Due to the complexity of the immune response to viral vaccination and infection, non-animal alternatives are not yet available for this project. However, laboratory work will be undertaken before moving into animal studies (e.g. to achieve optimal expression levels of protein from DNA vaccines).

Why were they not suitable?

See above.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers to be used are estimated by projecting the likely number of studies to be performed during the five year duration of this licence from the current demand, which is likely to be at a peak due to the current COVID-19 pandemic situation. Typical experiments will have at least one, possibly up to three treatment groups and a control group with up to around 10 mice per group a likely maximum.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of animals being used over the course of this project was estimated following a literature search for experiments typical of those to be performed. Prior to designing individual experiments to be performed under this licence, in conjunction with using the NC3Rs Experimental Design Assistant, a systematic literature review will be performed (I have undertaken systematic review training) to identify relevant studies (e.g. studies using a similar vaccine presentation or involving challenge infection with the same virus) in order to obtain the best data possible on effect sizes (for example) to inform study design and power calculations.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where animals remain naïve at the end of an immunogenicity study (i.e. control animals), the possibility of sharing tissues with research colleagues will be explored. The facility electronic distribution list and internal intranet notice board can be used to alert colleagues in advance of the availability of fresh tissue for ex vivo use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Adult mice will be used as they are widely used models of viral infections with a broad range of reagents available to measure immune responses to vaccination and infection.

Why can’t you use animals that are less sentient?

Animals at a more immature life stage, species that are less sentient or animals that have been terminally anaesthetised are not appropriate alternatives because immunisation requires a fully developed and functional immune system for the development of protective immune responses and efficacy studies require clinical disease to be manifested in control animals to demonstrate a reduction in infection in treated or vaccinated animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The welfare costs to animals will be minimised by the provision of group housing with environmental enrichment, bedding and or nesting material as standard. Animals will be handled and restrained with the minimal effective restraint - the use of the tail for handling and restraint will be minimised.

The potential for pain and inadvertent infection will be minimised by use of single use sterile needles of the appropriate size for the procedure. Local or general anaesthesia will be applied, where appropriate, under the direction of the Named Veterinary Surgeon. Injection sites will be clipped or shaved and cleaned ahead of injection to minimise the risk of contamination and to allow regular monitoring of the injection site after the procedure. Veterinary intervention will be promptly sought as required and appropriate humane endpoints applied in the unlikely event that adverse effects develop and cannot be controlled. Full records of procedures undertaken, daily monitoring and veterinary requests

will be maintained using both an established electronic facility management software and / or hard copy records.

All licensees and animal care staff will be trained, supervised and signed off as competent for the procedures they will undertake.

Where necessary, supportive husbandry will be provided for mice that have been infected with virus, for example mash and additional warmth and nesting material for mice that become lethargic and/or inappetant.

For studies involving infection with a strain of virus not previously used, a preliminary study will be conducted with enhanced monitoring to characterise the time course of adverse effect progression to ensure that appropriate monitoring and application of humane end points can be applied at a point that minimises the risk of avoidable suffering. This will include out of hours monitoring if required as a result of rapid progression of infection.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

LASA and NC3Rs best practice guidelines will be followed in respect to refining blood sampling and injection techniques. The NC3Rs grimace scales will be used to monitor mice for potential signs of distress. Home Office and FELASA severity information will be used to ensure that the actual severity experienced by the animals can be recorded and limits within this licence adhered to. Body condition score charts specific to the mouse will be used.

Home Office Code of Practice will be used to ensure animal care and housing is appropriate. This guidance will be used in conjunction with the advice available in the NC3Rs Resource Hub for both housing and handling of animals.

The NC3RS Procedures with Care resource will inform personal licensees of refinements in the conduct of the minor procedures undertaken in this licence.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I receive regular e-mail updates from University colleagues including the Named Training and Competency Officer (NTCO). I am registered for alerts on the NC3Rs website and follow @NC3Rs on Twitter, this alerts me to the latest developments, for example in non-aversive mouse handling. I will continue to communicate with colleagues at the University and other institutes that are conducting similar studies to find out about the latest refinements they have introduced.

64. The Development and Plasticity of Functional Properties of Brain Cells

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Brain, Functional cellular properties, Neurons and glia, Development, Plasticity

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this programme is to gain new knowledge about how the functional properties of a brain cell are established during development and how these properties might change as a result of brain activity.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Neurological disorders, such as epilepsy, autism and schizophrenia, are associated with alterations in the functional properties of brain cells. These functional properties are crucial in determining how individual brain cells transmit information to one another. In the case of neurons in the brain, important functional properties include the type, number and strength

of synaptic connections that the neuron forms with other neurons, plus the electrical properties that determine how the neuron transmits action potentials. In the case of glial cells such as astrocytes, key functional properties include intercellular communication via gap junctions, plus intercellular chemical signalling that regulates the local cellular environment.

Alterations to functional properties of brain cells can result from aberrant events during development, changes to brain cells during a person's lifetime, or a combination of both of these processes.

Therefore understanding the mechanisms by which brain cells establish their functional properties is crucial if we are to understand disease processes and generate new avenues for effective treatments. To advance our understanding, we require a much greater understanding of how the functional properties of brain cells are controlled by their genetic information (i.e. cell intrinsic information) and how much is influenced by ongoing signalling between cells as the brain is developing (i.e. cell extrinsic information). The current work is therefore important as it will provide new knowledge about how the functional properties of brain cells are hard-wired during development and how these properties can change as a result of the brain’s activity.

What outputs do you think you will see at the end of this project?

- The output will be the advancement of scientific knowledge about the genetic and environmental factors that control how the functional properties of brain cells are established and can be altered by activity within the nervous system. The project will produce the following outputs:
- Publications describing novel findings on the functional properties of brain cells that are pre- determined during development.
- Publications describing novel findings on the functional properties of brain cells that are plastic and altered by the brain's activity.
- Datasets on functional properties of brain cells that can be incorporated into computational models of normal and abnormal brain function.
- Advances in understanding of how environmental and genetic factors may influence brain cells in individuals with neurological disorders such as epilepsy, autism and schizophrenia.

Who or what will benefit from these outputs, and how?

In the shorter term:

- The outputs and advances in our understanding of brain cell properties will benefit a large national and international community of researchers working on mammalian brain development and plasticity.

- The outputs and advances will also be of interest to an even wider community of researchers in neuroscience, pharmacology, psychology and psychiatry, including those researching the causes and consequences of neurological disorders.

- The outputs will also benefit computational scientists, who are aiming to produce realistic simulations through which they can better understand brain function in health and disease.

In the longer term:

- The outputs and advances in our understanding of brain cell properties will benefit parties (e.g. the pharmaceutical industry) that wish to identify molecular targets for new products that could improve neural functions in neurological disorders.

- Also, they stand to benefit clinicians who seek to treat patients, or better explain disease processes to patients and their carers.

How will you look to maximise the outputs of this work?

- By publication of the resulting data on "preprint" servers and in peer-reviewed international journals.

- By contributing to the UK knowledge economy through timely publication of high-quality research accessible to academics and clinicians.

- By sharing the data (published and unpublished) through presentations at conferences, as well as at specialist user groups (e.g. clinicians, industry).

- By sharing the raw data and computer code on publically-available repositories.

- Through public engagement activities, such as school outreach and public-facing events.

- By maintaining and developing existing links with the pharmaceutical industry.

- By contributing to the economic competitiveness of the UK through enhancement of researcher career development and the training of young scientists.

Species and numbers of animals expected to be used

- Mice: 19600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

- This research programme will use mice across a range of life stages (from embryo to adult) for the following reasons:

- The mouse brain is a simple model of the human brain, but significant advances in our understanding of neurological disorders are underpinned by studies on small animals.
- The anatomy and physiology of the mouse brain is well described, and previous work provides an important foundation for the research.
- The experimental methods that will be used have been specifically designed for, and successfully used in, mice.
- The project will use a range of life stages, from embryo through to adult, because a large proportion of the project is to study the development of brain cells. Most brain cells are generated before birth, their functional properties emerge as the animal grows during the late embryonic and early postnatal stages, and then their functional properties reach maturity during later postnatal stages.

Typically, what will be done to an animal used in your project?

Mice used in this research programme will experience the following:

- Genetically altered animals will be bred, which is not expected to cause any harm.
- On occasions, animals will be treated for up to two weeks with drugs, the majority of which will have proven tolerability and safety in animals and humans.
- To study early brain development, some embryos will have minute amounts of substances delivered to their brains. This requires a surgery to be performed on a pregnant female under general anaesthetic, and the offspring are typically monitored for up to six weeks as part of the experiment.
- Some animals will have substances delivered to the brain or have miniature recording devices placed on, or in, the brain. This requires a surgery to be performed on the animal under general anaesthetic, and the animals are typically monitored for up to four weeks as part of the experiment.
- Some animals will have their heads temporarily fixed in place for brief periods of time, so that their brain activity can be monitored whilst they are awake. Mice are continuously monitored during these sessions, which typically last no more than three hours. The mice are fully habituated to the apparatus beforehand and would typically experience either one session, or five sessions over a week.
- At the end of experiments, animals may be recorded under terminal anaesthesia, will be killed by humane methods, and cellular measurements will typically be performed in post-mortem tissue. This is not expected to have any welfare impact on the animals.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice in this research programme may experience adverse effects that include the following:

Animals that undergo surgery are expected to experience some pain and discomfort, but recovery is expected to be uneventful. All surgical procedures are carried out under aseptic conditions and with analgesia always provided during the surgical procedures and continued until all signs of discomfort have disappeared.

Animals that have their heads temporarily stabilised might initially experience brief periods of frustration (over minutes), but this is only expected to have a minimal impact upon wellbeing and the animals will be continuously monitored and carefully habituated to the apparatus.

Some animals may experience a modest decrease in body weight, due to a change in sleep-wake pattern or due to administration of an agent that regulates gene expression. These animals will be frequently monitored and weighed, and offered additional palatable food, as required.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice:

60% sub-threshold

10% non-recovery

17% mild

13% moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals are needed to achieve the aim of this project because they provide the best way to study the functional properties of brain cells. The functional properties of a brain cell depend heavily upon the type of cell, its developmental lineage, where the cell is

positioned within the brain, how the cell interacts through electrical and chemical signals with other cells, and the brain's activity patterns. The brain's activity reflects a combination of internally- and environmentally-generated signals and animals offer the only way to examine the importance of how the brain's activity changes over the course of an animal's lifetime and varies depending on the brain state, such as sleeping and waking states.

Which non-animal alternatives did you consider for use in this project?

- Computational models of the development and plasticity of brain cell functions.
- Cultured cells, comprising either immortalised cell lines or brain cells derived from induced pluripotent stem cells.

Why were they not suitable?

- Although computer models can provide insights into the behaviour of individual neurons and simple networks of connected neurons, the models lack the complexity to address the key research objectives here.
- Whilst important for some types of investigation, cells grown in culture are either unable to exhibit, or fail to acquire, many of the key functional properties of brain cells. Cultured cells lack tissue organisation, exhibit abnormal interactions with one another, and the patterns of activity in cultured cells do not recapitulate those observed in the intact brain. In the case of brain cells derived from induced pluripotent stem cells, these lack the normal connectivity and activity of the intact brain, and are known to lack mature functional properties.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is based upon extensive experience of the technological approaches and experimental design required to address the scientific objectives, which has been acquired by performing related projects.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Steps taken to reduce the numbers of animals in this project include:

- The use of experimental designs that incorporate statistical analysis and randomised, blinded experimental methods.

- Pre-emptive use of power calculations to establish the minimum number of animals required to achieve a meaningful result.

- Wherever possible, statistical sensitivity will be increased with experimental designs that compare experimental and control cells from the same animal. This statistical 'pairing' can be used with more powerful statistical tests, which can reduce the number of animals.

- Multiple recordings will be performed to maximise the number of data points per animal. Previous evidence indicates that data from different cells in the same brain can be treated as independent values, which is critical for the application of parametric and non-parametric statistics.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- The use of well-managed and conservative genetic mouse breeding strategies and the use of both male and female mice, as well as heterozygote mice where appropriate.

- The use of methods that detect the activities of multiple brain cells in a single animal, thereby reducing the number of animals required, and at the same time generating large biological datasets to capture the complexity of the neural circuits.

- The use of extensive literature searches to avoid replication of experiments and pilot experiments before embarking on full-scale studies.

- The incorporation of data into computational models to aid further hypothesis development and prediction testing.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

- The use of genetically altered mouse lines represents a refined method for selectively labelling and manipulating specific populations of brain cells. These mouse lines are not associated with any adverse effects.

- The delivery of substances to the brain during either embryonic or postnatal life, represents the most refined method to investigate brain cells with a defined lineage, at a specific time point in development, and without complications from causing changes to other parts of the nervous system or organism. This method alleviates the need to

generate new and complex transgenic mouse lines, which requires breeding many generations of mice.

- The use of miniature devices and periods of head stabilization, represents the most refined method for monitoring and manipulating brain cell function in the non-anaesthetised, intact brain. The activity of brain cells is measured using fine microelectrodes and minimally-invasive imaging fluorescence microscopy techniques. This approach is also compatible with the most refined methods for targeting/manipulating genetically-defined populations of cells, using techniques called optogenetics and chemicogenetics.

Why can't you use animals that are less sentient?

Animals that are less sentient:

- Do not have the complex neural tissue, brain cell types and connectivity that are representative of the mammalian brain.
- Do not follow common principles of brain development that is characteristic of mammals.
- Generally have nervous systems that are much less studied, and so there is not the knowledge foundation or databases to support the current project.
- While some questions can and will be addressed using terminal anaesthesia, certain aspects of brain cell function can only be studied if the animal is awake.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Procedures will be refined through the following general approaches:

- Health monitoring of adverse effects with humane endpoints, and in a manner that minimises cumulative effects of experimental steps.
- The use of peri-operative analgesia, which will continue after surgery for as long as required to alleviate any pain.
- The optimisation of experimental parameters to produce reproducible effects with the minimum intervention.
- The use of the existing literature and databases to aid selection of cell labelling and manipulation reagents.
- The incorporation of advances and refinements in technologies, including recording devices.
- The use of feeding regimes that reduce post-operative weight loss and speed post-operative recovery.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Experiments will follow best practice guidance from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (https://nc3rs.org.uk) and from the Laboratory Animal Science Association on aseptic surgery and the administration of substances (https://www.lasa.co.uk).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The primary establishment creates several forums for sharing advances in the 3Rs, in the form of departmental animal welfare meetings and the Animal Welfare Ethical Review Body (AWERB) meetings. Represented at these meetings are the vets, NACWOS and the regional programme manager for the 3Rs, all of whom provide updates and advice on refinements.

65. Nervous System Modelling, Protection and Repair

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:

(i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

peripheral nerve, CNS cell culture, tissue engineering, advanced therapies, nerve repair

Animal types	Life stages
Mice	adult, neonate, embryo, juvenile, pregnant
Rats	adult, neonate, embryo, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To improve understanding of how the nervous system responds to damage and disease and to develop and test new ways to treat patients with nervous system injuries. This involves developing tissue engineering, cell, drug and gene therapies and creating advanced cell culture models.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Nervous system damage due to injury or disease can be debilitating, causing physical disability and pain as well as impacting on personal and societal interactions for those affected. Understanding damage and promoting repair in the nervous system are key areas of biomedical research. Recent advances in regenerative medicine and the combination of physical and biological sciences to understand tissue damage and repair mean that this field is advancing rapidly. Translation of new nervous system repair technologies to the clinic requires laboratory models to understand cellular and molecular mechanisms underpinning damage and repair, both in vitro and in vivo. To progress new treatments, regulators require a comprehensive set of experimental data to support the application.

Our work involves the discovery and development stages of new treatments for nervous system damage and depends on the availability of robust in vivo and in vitro models.

What outputs do you think you will see at the end of this project?

The overall benefits of the project are:

1. Improved understanding of nervous system damage

2. Identification, testing and development of therapies to treat nervous system injury

These benefits are likely to include new knowledge, publications and treatments and will be achieved through (1) the use of in vitro CNS models to explore cellular responses to damage and to screen potential therapies, and (2) the use of the nerve repair model to develop and test new approaches to treat nerve injury.

The benefits will directly contribute to improving the understanding of nervous system injury and the development of therapies. The cell culture aspects also serve to provide innovative new tools for neuroscience research, screening and testing which are likely to be adopted by other users such as industrial and clinical researchers. An established non-animal approach in our lab is the use of mathematical modelling to simulate new nerve repair interventions, so an additional output from this work is to inform and parameterise in silico models which are powerful tools to accelerate development of therapies and reduce reliance on experimental models.

Long term consequences are likely to include improved clinical treatments for nervous system injury and disease, improved functional outcomes, reduced reliance on nerve autografts, fewer side effects, novel neuroprotective treatments and accelerated development of new therapies.

Who or what will benefit from these outputs, and how?

In the long term the people who will benefit from better treatments for nervous system damage are the patients affected by these injuries, their families, friends and colleagues. Our engagement with patient communities has identified the current lack of effective

treatments to improve outcomes after injury as being a priority to improve their health and wellbeing. Improved treatments will enable injuries to be treated more effectively with fewer side effects, better outcomes, faster return to work, reduced mental illness and reduced cost to those affected and the healthcare and welfare systems.

Clinicians and other healthcare workers will benefit by having more effective treatments to offer patients, improving outcomes, reducing disability, shortening rehabilitation times and reducing complications and side effects.

In the short and medium term, researchers working on the project will benefit from improved training and understanding, career development, and acquisition of advanced transferable skills. The wider research community will benefit through increased understanding of the progression and treatment of nervous system damage.

How will you look to maximise the outputs of this work?

Data will be shared directly with our academic and clinical colleagues, published and disseminated through conferences and networking and will underpin future collaborative projects with other research groups, industrial partners and clinicians.

Open-access publication will make the scientific outputs available freely to all.

Datasets from in vivo experiments will be made available to the global nerve injury research community via an open source data repository.

New therapeutic approaches that show promise will be taken forward for clinical development using our established translational pipeline involving institutional support structures for IP protection, engagement with regulators, clinicians and patients, and commercialisation.

Species and numbers of animals expected to be used

- Mice: 400
- Rats: 2900

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rats and mice are used because they are the most appropriate least sentient option that have the required anatomical and physiological features for this work.

For CNS cell culture the brains from neonatal animals will be used because these provide a viable source of mammalian cells at an appropriate stage of development for use as in vitro models.

For nerve injury models in vivo adult rodents tend to be used. That is because they recapitulate the key features of adult human nerve injury and regeneration. The nerves in the hindlimb of a rat are of similar dimensions to the nerves in human hands, which means they can be used to model clinical microsurgical repair scenarios directly using equivalent approaches and instruments.

Typically, what will be done to an animal used in your project?

For CNS cell culture, animals will be killed and then brain tissue removed to provide a source of living cells.

For nerve injury, typically an animal will be anaesthetised then a nerve in one hindlimb will be exposed, damaged (e.g. crushed or cut), then repaired microsurgically. A treatment being investigated (or appropriate control) will be applied and then the skin will be closed and the animal will regain consciousness and be returned to its home cage and maintained for a period of a few weeks to months. The duration will depend on what is being studied, for example initial responses to damage may be measured in the first few days, neuronal growth through the injury may be measured after 2-4 weeks and recovery of function can take 3-4 months. During that time the animal will be monitored for signs of adverse effects and the recovery of nerve function may be measured. At the end of the experiment the animal will be placed under terminal anaesthesia and nerve regeneration assessed using electrophysiology, then tissues removed post mortem for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

For the nerve injury model the impact on the animal is loss of sensation and movement in one hind paw. Depending on the injury/treatment, this functional loss will gradually recover during the weeks following the experiment. Animals recover consciousness within minutes following anaesthesia and are able to move around their home cage and can eat, drink and interact normally.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For all animals in the nerve injury experiments the expected severity is moderate. For the animals used as a source of CNS cells the expected severity is mild.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

(1) Before a nerve injury treatment can be considered for clinical use it is essential to assess it in a living mammalian system. Initial development can be conducted using non-animal systems, but it is not possible to progress a new treatment to the clinic without conducting tests in animals. There are currently no alternatives which model all the body systems that would potentially influence outcome.

(2) Cells derived from freshly culled animals are essential for CNS models, however, it is important to note that the resulting cell culture systems are an alternative to live animals in many experiments. Alternatives include fresh human cells, cell lines and various stem cell sources, but in some cases these options are not suitable; cell lines and differentiated stem cells can fail to resemble natural brain cells, and fresh human brain cells are difficult to obtain and grow in culture.

Which non-animal alternatives did you consider for use in this project?

(1) We use non-animal alternatives to model nerve injury wherever possible and these include sophisticated computational and in vitro approaches, including 3D cell culture and engineered tissue models.

(2) Alternatives to rodent CNS cells include fresh human cells, cell lines and various stem cell sources.

Why were they not suitable?

(1) Our computational and in vitro models are good at predicting some of the outcomes of injury and treatment but currently they cannot integrate all of the different body systems that are likely to have an influence.

(2) Non-animal cell sources are used wherever possible but in some cases these options are not suitable; cell lines and differentiated stem cells can fail to resemble natural brain cells, and fresh human brain cells are difficult to obtain and grow in culture.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers are estimated based on experience of similar work conducted by the research group over the course of previous projects. Each experiment is designed to provide scientifically robust data from the minimum number of animals. Most of the animals included in these numbers are used for breeding approximately (1300 rats and 200 mice) with smaller numbers used in nerve injury approximately (900 rats and 100 mice) and CNS cell culture approximately (700 rats and 100 mice) over the course of 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

(1) For nerve injury, extensive rounds of in vitro and computational modelling are conducted in advance, allowing treatments to be developed to an advanced stage before testing in animals.

Where appropriate, power analyses are performed to determine the minimum number of animals in each experimental group required to provide statistically significant results. This is facilitated by previous datasets that provide an indication of the standard deviation and the relevant effect sizes for the outcome measures. Tools such as the online Experimental Design Assistant (NC3Rs) can be used. The research team is skilled in power analysis and experimental design and where more specialist expertise is required the advice of a statistician is available on site. Randomisation and blinding are incorporated into the design of experiments to reduce bias.

(2) For CNS cell culture, these are prepared only when specific experiments are undertaken and animal numbers can be calculated based on the number of cells required. Typically up to a litter of pups would be culled at one time and their cells pooled and used for multiple experiments to maximise data and minimise variability

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Multiple outcomes are investigated in the same animal and our methods for sensitive quantification of neuronal regeneration enable significant data to be obtained from small numbers of animals per group. Where possible, additional information about the time course of regeneration will be obtained using repeated measurements on the same animal. This further reduces the number of animals required by avoiding having to set up multiple groups with different end point times. Results are compared between groups, normalised against contralateral controls, and compared to other devices or controls. The

standardised nature of the approach means results can be compared directly with previous datasets, minimising the need to include additional groups.

Computer modelling will be used to simulate specific nerve injury and repair scenarios where possible and the outcomes from these models will be used to optimise any subsequent experimental approaches. Where breeding is required, an efficient approach will be used to minimise the number of animals required. Because nervous system damage can affect both sexes, male and female animals will be used in experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

(1) Nerve repair in rats and mice under general anaesthesia is the least severe procedure that enables a reliable assessment to be made in an environment that resembles the human repair situation. Various animal models have been used for peripheral nerve repair research and the rat has become established as the species of choice for gap repair while mice are used where genetic alterations are required. These models are designed to minimise animal suffering and provide valuable preclinical data. The sciatic nerve is the biggest nerve in the animal and is readily accessible with minimal disruption to surrounding muscles; it is the only easily accessible nerve with a long enough section suitable for gap repair; in rats it is very similar in terms of size to human digital nerves enabling devices of clinically relevant dimensions to be tested; loss of function in a limb does not excessively reduce the ability of an operated animal to feed and drink or move around.

(2) For CNS cell culture, rats and mice are the lowest vertebrates whose CNS cells have been characterised thoroughly and show a similar response to damage as the human (e.g. glial scarring). Furthermore, the availability of genetically altered animals with markers such as fluorescent proteins increase the value of using rats and mice for this work.

Why can’t you use animals that are less sentient?

The animals chosen are the least sentient option for the required experiments. More immature life stages and less sentient species are not suitable for use in these studies

because they do not recreate key features of the adult human nervous system aspects needed to be present in the models.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Surgical procedures are accompanied by administration of analgesia and regular monitoring to detect any signs of pain associated with the procedure.

In some experiments it may be possible to reduce severity further through transecting and repairing one of the distal branches of the nerve rather than the main nerve trunk, or through transient crushing of the nerve rather than complete transection. These approaches are used when appropriate to reduce the extent of damage caused by the injury.

Where it is beneficial to reduce a potential immune response to an implanted material (e.g. containing human cells) then animals can be immunosuppressed using drugs, or alternatively immunocompromised strains will be used to minimise the potential harm caused by the additional handling and injections associated with drug administration.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Licensees will be directed to published best practice available via the NC3Rs resource library when planning experiments. Relevant available guidance includes topics such as welfare assessment, anaesthesia, aseptic technique in rodent surgery and administration of substances, as well as the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery.

PREPARE guidelines (norecopa) provide a useful checklist to be used when planning new experiments and ARRIVE guidelines will be followed during reporting of results.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The researchers will remain actively involved in the 3Rs research community and will attend workshops and symposia and monitor the NC3Rs gateway and e-newsletters and new literature to stay informed about advances in the 3Rs. Opportunities that are identified for further refinement will be discussed with the Named Persons and animal unit staff as well as within the research group and, where it is appropriate for an approach to be changed, this will be communicated to all licensees by the PPL holder.

66. Motor Neuron Development and Disorders

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Motor neurons, Axon guidance, Zebrafish, Motor neuron disease, Congenital Cranial Dysinnervation Disorders

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, adult, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The single main purpose of this project is to elucidate mechanisms that guide the axon growth and connections of motor neurons, and to unravel how abnormalities of these mechanisms can lead to disorders. The study will incorporate detailed description of the ways in which axons project to their target muscles, as well as manipulations of gene and protein functionto elucidate the molecular mechanisms that underlie axon guidance. Genetic models will be used to identify how particular mutations lead to motor neuron disorders and the effects on neuroanatomy and behaviour will be analysed.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The mechanisms by which axons grow and find their targets are poorly understood. Motor neurons are a key neuronal group that are responsible for locomotion and other animal behaviours. Gaining insight into the mechanisms of axon guidance and its molecular underpinnings will allow us to elucidate the aetiology of disorders. The manipulations of genetic and molecular function that we propose will give insight into the role of genes during normal processesof axon guidance and connectivity. As well as illuminating developmental processes, these models will provide insights into neurodevelopmental and neurodegenerative disorders of motor neurons.

What outputs do you think you will see at the end of this project?

The project outputs will be scientific papers and conference presentation. We will share data with relevant other research groups and collaborators and data and technical insights will be shared on platforms such as ZFIN. Any reagents or transgenic lines will be shared with the research community via relevant repositories after publication of our findings.

Who or what will benefit from these outputs, and how?

Beneficiaries of the work will be immediate colleagues within our establishment and those in the neuroscience and developmental research community. This includes basic researchers interested in mechanisms of axon guidance and the genes involved, and those interested in how insights into the genes and proteins studied can translate into other systems. Other beneficiaries include clinicians and scientists studying neurodevelopmental and neurodegenerative disorders that affect motor neurons. In particular, our work has direct implications for studies of eye movement disorders, which are relatively common in the human population with squint having a prevalence of 1%. Whereas squint is not a life- threatening condition, it produces difficulties for sufferers, and can be very debilitating. We will share our findings with clinicians in this area, which provide insights for screening of patient DNA, and for approaches to therapies. We also plan to work on mechanisms of causation of motor neuron disease (MND: incidence ~2.5 persons per 100,000). Unravelling the contribution of several genes to MND would eventually benefit patients through improved diagnosis and treatments.

How will you look to maximise the outputs of this work?

The outputs of our work will be datasets consisting of imaging data defining phenotypes in the developing zebrafish. Behavioural assays will also be used to analyse eye movements and locomotion. These data will be shared with other scientists in our University, nationally and internationally, published in peer-reviewed journals and presented at conferences. We will also share information with clinicians to improve patient care and to inform clinical studies to identify mutations in human populations

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 13,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Zebrafish will be used for this project and the majority of our analyses will involve experiments performed on larvae before the 5 dpf timepoint at which these animals become protected. Moreover, zebrafish have a large number of advantages for studies of nervous system development and disorders. They can be imaged readily, are genetically tractable and exhibit measurable behaviours from 2 or 3 dpf, and a number of transgenic lines are available. In the case of studying motor neurons, the motor systems have similar properties to those of humans, including the ocular motor system and the segmental arrangement of spinal motor neurons. Hence studies in the zebrafish can provide very useful information about genetic function and the aetiology of motor neuron disorders in humans. We will examine various wild type and transgenic zebrafish lines. We will breed adult fish and collect naturally spawned embryos in order to analyse phenotypes in larval stages, typically 1-5dpf. Zebrafish have several advantages over other model organisms. Large numbers of embryos can be obtained from each breeding pair, larvae can be treated using water soluble pharmaceutical agents for high- throughput drug screening and are translucent making them easy to image and analyse.

Typically, what will be done to an animal used in your project?

We will maintain various wild-type zebrafish lines, lines carrying mutations and transgenic lines. We will use cutting edge techniques, such as CRISPR/Cas9, to generate genetically altered models of neurodevelopmental and neurodegenerative diseases. Most breeding of freshwater teleost lines is undertaken by natural spawning following pairing of male and female fish, but in some cases frozen sperm and in vitro fertilisation will be used, yielding embryos which are then allowed to develop.

Skin swabbing or fin-clipping will be used in some cases. This is because, for some of the transgenic or mutant lines received or produced by this project, identification of carriers from outcrosses is only efficiently achieved by PCR amplification of DNA extracted from surface mucus/a small tissue sample. This is routinely done in zebrafish by swabbing the skin or section of a small portion of the caudal fin of young adults, under sterile conditions and light anaesthesia. A shortened caudal fin does not affect the swimming abilities of the fish and regenerates extremely well.

In order to analyse alterations in nervous system development, function or viability, behavioural analyses will need to be performed. In the majority of cases this will involve non-injurious filming of juvenile or adult fish in environments where they arepresented with

specific visual stimuli, or in which their swimming patterns in response to certain stimuli are monitored.

What are the expected impacts and/or adverse effects for the animals during your project?

In the vast majority of cases, the mutations and transgenes carried by the adult fish are unlikely to have any harmful effects (subtle morphology changes which have little or no effect upon viability and health). However for some mutations, the fish may develop defects in neural development, which might lead to behavioural abnormalities. In other cases, fish models of motor neuron disease might lead to compromise of axon development and neuronal death. This could in turn lead to muscle atrophy and loss of movement. Whereas these changes would entail adverse effects on the larvae, we plan to analyse the animals before 5 dpf. Adults would not be kept as genotypes which are likely to yield such adverse effects.

Any viable transgenic animal that shows harmful abnormalities such as inability to feed or swim will be culled by schedule 1 methods as soon as the abnormality is apparent. This also applies to juveniles and larvae.

Immobilisation of fish for eye movement or neuronal activity analysis may cause minor distress or discomfort but most tests are likely to only last a few minutes and fish will be killed by schedule 1 procedures at the end of the testing.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities of the majority of procedures (95%) to be undertaken is mild. However, for a few transgenic lines (5% or less), there is a possibility that some genotypes will develop neurodegeneration leading to a severe assessment.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of animals is necessary for this project as the axon guidance of neurons, formation of neural circuits and analysis of motor neuron degeneration requires insights in

the intact animal, including the interaction of neurons with their environment. The zebrafish is an optimum model for our approaches due to its huge advantages for imaging and genetic studies. Our procedures our unlikely to generate pain or distress. In the rare event that the fish need to be kept at the end of a procedure, it is first monitored for sign of pain/distress (lack of activity, appetite or sexual behaviour). Any individual presenting any sign of pain or distress that is not rapidly curable will be killed under schedule 1.

Which non-animal alternatives did you consider for use in this project?

Recent work has been undertaken to elucidate the mechanisms required to differentiate ocular motor neurons from stem cells in rodents, which could potentially give an in exhaustible source of experimental material. Thus far, these protocols are inefficient and generate very few relevant cells. However, the majority of our work over the years has focussed on early stages of development, before these species are protected. We have used in vitro approaches, which although they involve the culling of animals to generate neurons, can give very large numbers of datasets from a single animal, in order to identify mechanisms and molecules that are involved in axon guidance processes and disease. Only then do we perform analyse in whole animals (larvae) in order to perform critical experiments, e.g. transgenesis that involve an intact environment.

Why were they not suitable?

As mentioned above, we are not yet able to use cell lines because they are not available or reliable. However, we have sought to reduce our animal use as much as possible in our projects as outlined below, by performing only the most critical experiments in intact animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The principle of reduction is applied for all procedures used. Number of embryos and adults used are defined statistically as the minimum required to ensure results. An indication of these numbers isgivenby starting numbers of embryos in most protocols being 100-150. Microinjection of these embryos would be followed by incubation to later developmental stages, with mortality of around 10%. Staining and imaging procedures would yield a sample of around 30 fish with 15 giving good images. The experiment would be repeated 3 times per condition. These procedures should yield a

sample size of around 45 per condition, with statistical analysis using non- parametric tests (Mann-Whitney).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Creating stable transgenic zebrafish lines is by itself a way to reduce number of animals used. This method allows us to test the effects of gene manipulation readily, reducing the number of animals which need to be bred for analysis, as transient gain and loss of function approaches require many more animals. In addition, transgenic fish with fluorescence for analysis of axon pathways minimise the need for staining protocols.

In studies over several years, we have optimised analyses of neuroanatomy and behaviour in order to reduce the number of animals needed. For example we are able to extract multiple metrics from images of axon pathways in order to achieve significance.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The minimum number of wild-type and transgenic zebrafish required for optimal breeding will be maintained. This will be determined from our extensive experience using this model organism. We will perform initial pilot studies, such as using transient genetic alteration techniques on small numbers of animals to determine their relevance in modelling either neurodevelopmental or neurodegenerative disorders. This will allow us to determine which genetic modifications to establish as stable zebrafish lines, and therefore optimise the number of animals to be used in the project.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use zebrafish as a model for neurodevelopmental and neurodegenerative disorders in this project. Most experimental procedures to be undertaken are unlikely to cause distress or harm to the animals. It is possible that some genetic alterations may affect the ability of larvae to swim freely. All animals will be closely monitored for signs of pain/distress and culled using schedule 1 methods if the effects are not rapidly curable.

For some of the transgenic or mutant lines received or produced by this project, identification of carriers from outcrosses is only efficiently achieved by PCR amplification of DNA extracted from a small tissue sample. This is routinely done in zebrafish by section of a small portion of the caudal fin of young adults, under sterile conditions and light anaesthesia. A shortened caudal fin does not affect the fish swimming abilities and regenerates extremely well.

In order to analyse alterations in nervous system development, function or viability, behavioural analyses will need to be performed. In the majority of cases this will involve non-injurious filming of juvenile or adult fish in environments where they are presented with specific stimuli, such as presenting fish with a rotating drum with vertical stripes and filming their eye movements, in order to measure the optokinetic reflex.

None of these procedures cause pain, suffering or distress to the animals, apart from in a very low proportion of animals when using models for motor neuron disease, as explained above.

Why can’t you use animals that are less sentient?

We will use zebrafish as they are a less sentient model organism compared to other vertebrate species. The majority of procedures will be restricted to larval stages of development (1-5dpf), in order to further reduce the sentience of the model used. Certain protocols, such as examining the optokinetic reflex or startle responses require intact and fully conscious organisms. However, where appropriate, other procedures, such as imaging, will be performed following terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For some of the transgenic or mutant lines received or produced by this project, identification of carriers from outcrosses is only efficiently achieved by PCR amplification of DNA. We have optimised all protocols including DNA extraction following fin-clipping to maximise yield and remove the smallest piece of fin possible. Where appropriate, we will transition to using skin swabbing as a more refined method for genotyping following optimisation. Animals are monitored following the clipping procedure. Fish are checked each day (2 - 3X) and monitored for any changes or signs of ill-health. Animals embedded in agarose for behavioural tests are culled immediately after testing.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will continue to engage with the most relevant literature using zebrafish as model organisms for disease to ensure that the numbers of animals used are in line with, but do not exceed, those publications. We will continue to consult the information available

through the NC3Rs to ensure that our animals receive the highest standard of care and can implement any required changes to our animal welfare practices.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive regular updates from the NC3Rs which we will engage with and look to implement any relevant changes to our animal welfare/facilities. Our establishment has introduced a yearly award to encourage research into the 3Rs and their implementation. We will continue to work closely with our animal technicians and NACWO to ensure that our zebrafish experience the highest standard of care.

67. Exploring the Mechanisms of Tertiary Lymphoid Organ Formation

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Salivary Gland, Inflammation, Autoimmune, Tertiary Lymphoid Organs

Animal types	Life stages
Mice	juvenile, adult, neonate, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to examine the role of tissue resident fibroblasts in the formation and maintenance of tertiary lymphoid organs in chronic inflammation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Inflammation is the process by which the body's own immune cells enter a target tissue, such as the salivary gland, gut or skin for example. This process occurs in response to infection, injury or where the body's immune system reacts to healthy tissue in a process called autoimmunity. When inside the tissue these immune cells can aggregate together in structures called tertiary lymphoid structures or organs (TLOs). We see these structures in

the tissues affected in certain diseases such as Sjogren's Syndrome. How and why these structures form and their exact role in inflammatory tissue pathology is poorly understood. If we can understand why and how these structures form we may be able to develop novel drugs to treat this disease.

What outputs do you think you will see at the end of this project?

We anticipate that this project will lead to the generation of novel data which will give rise to mechanistic insights into diseases with a significant unmet clinical need.

Project outputs will include:

- advances in scientific knowledge on the role of fibroblasts in autoimmune salivary gland and joint inflammation.
- scientific and lay publications of our findings and methodology.
- presentation of our data and/or methodology to the wider scientific community through conference oral and poster presentations.
- identification of novel therapy targets or agents which can be taken forward to develop new therapies.

Who or what will benefit from these outputs, and how?

Short-medium term

Scientific developments and innovations leading to our enhanced understanding of the role of immune effector fibroblasts on the formation of tertiary lymphoid structures will have an immediate impact on the ongoing projects at the host organisation, and also further afield upon dissemination. Moreover, the project aims to improve our understanding of the processes driving pathology in chronic disease, specifically disease pathways mediating the formation and persistence of tertiary lymphoid structures.

Medium-long term

We will disseminate our findings at national and international scientific conferences with the view of fostering further collaborations based on the concepts and ideas incorporated in this proposal. We envisage that these collaborations will occur during and following the completion of this project, and therefore represent a medium to long-term impact of this work. Using these collaborations, we will develop the infrastructure needed to translate these findings into clinical trials and ultimately, clinical practice, therefore fully exploiting the translational potential of this work.

Long-term

Clinical academics, pharmaceutical companies and patients directly will benefit from advances in our understanding of disease pathology along with identification of novel targets which can be taken forward to develop new therapies. We will develop collaborative networks to realise the translational potential of our findings over the subsequent 5-10 years following the completion of this project.

How will you look to maximise the outputs of this work?

Dissemination of information:

We will work with the relevant teams within our establishment to facilitate communications and resulting impact. We plan to use several routes to disseminate our findings to the wider scientific community and the public that will facilitate end-user engagement, namely:

(a) Peer-reviewed publication: we aim to publish high impact papers based on the findings generated from the research grants funding this project licence. In addition, our group has a strong tradition of publishing methodology papers and negative data to ensure that groups do not unnecessarily repeat experiments that either technically are flawed or biologically yield the null hypothesis.

(b) Presentations: we and our collaborators will present data at internal seminars along with national and international conferences, such as British Society of Rheumatology, European League Against Rheumatism Annual Congress and American College of Rheumatology.

(c) Dissemination via international societies: we and our collaborators are active members of various scientific societies including: European Workshop for Rheumatology Research and British Society for Rheumatology, allowing our findings to be disseminated to the wider scientific community in societal magazines and training workshops.

Enhancement of public understanding and engagement with research:

We will take advantage of several events organised by the Public Engagement Working Group at our organisation and local charities to facilitate the public's awareness of our research:

Lay Resources: we would develop lay resources, in collaboration with our patient/public research partners (PRP), for publication of Atlas of Science. Additionally, we will continue to involve patients and the public in the delivery and dissemination of research generated from this project.

I actively engage with our rheumatology patient research partners in all stages of our research including research strategy and planning, as well as dissemination to lay audiences. I also work closely with our CLUSTER consortium (https://www.clusterconsortium.org.uk) parent champions who are parents of children with

childhood onset arthritis. These partnerships are pivotal in developing our ongoing research strategy and ensuring our work is disseminated to lay audiences.

Clinical networks and translational collaborations:

My team and I are active members of several multi-institute research centres and will be able to present our findings at least twice annually at ongoing Centre seminars. We will also attend clinical conferences (e.g. European League Against Rheumatism, American College of Rheumatology, British Society of Rheumatology) where we will present our data and foster collaborative opportunities for translational research across the fields of rheumatology, ageing and chronic inflammatory diseases. As a team of both clinicians and scientists we have access to unique patient cohorts and therefore the expertise and ability to translate findings rapidly to early clinical studies.

Species and numbers of animals expected to be used

- Mice: 3000 over 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the best vertebrate model for the study of persistent disease because:

1. the main components of their immune system are shared by humans; this is essential where immune responses as opposed to the function of individual genes is being studied and thus will produce satisfactory results.

2. a wide range of wild type and genetically manipulated strains of defined genetic make-up are available

3. an extensive range of reagents is available for analysis of the cellular and molecular interactions occurring during immune responses

For the cannulation experiments we will use mice aged 8 weeks onwards. This is to ensure that their salivary glands are well developed and physically possible to cannulate.

Typically, what will be done to an animal used in your project?

Breeding and maintenance protocols:

Animals will be grouped or paired, mated and subject to such other non-painful procedures as may be required for the conventional breeding and maintenance of animals with specific genetic alterations or harmful mutations. Animals may be marked and genotyped

by appropriate husbandry methods which cause no more than momentary discomfort. Specifically this includes ear notching or the withdrawal of a very small amount of blood. Some animals will be humanely killed via a Schedule 1 method with tissues taken for analysis.

For cannulation experiments:

Whilst part of an experimental cohort, animals may be given substances via intraperitoneal, subcutaneous or intravenous injections, or by oral gavage. Blood samples may be taken according to recommended published guidelines via the saphenous vein (on a weekly basis) or via cardiac puncture under terminal anaesthesia. For the cannulation procedure, mice will be anesthetised using an injectable reversible anaesthetic. Once sedated, the opening duct of the salivary gland will be cannulated with a substance delivered directly into the gland. Cannulation may be repeated up to four times with animals humanely killed up to 28 days post-cannulation. Animals will be humanely killed via a Schedule 1 method or following a larger bleed performed under a terminal anaesthetic at the end of the experiment with tissues taken for analysis. Procedures will be undertaken using the most appropriate anaesthetic and analgesia will be given where appropriate. Any new substances or route of administration will be tested in a small pilot study and the mice monitored daily for signs of adverse effects.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals may experience pain and discomfort from administration of substances or blood sampling, but the discomfort is expected to be minimal and not long-lasting. Cannulation will result in localised pain and discomfort, so mice will anesthetised and analgesia given where appropriate. some weight loss is expected as the animals may refrain from eating and drinking after the procedure. This is expected to last for only one day after which a full recovery and return to normal intake is expected. Any animal showing deviation from normal behaviour as judged by body weight, body condition, general and coat appearance, gait or behaviour will be treated with pain relief (only if the adverse effect is considered to be due to pain) and further monitored. Before adverse effects exceed a moderate severity level, animals will be killed to prevent any ongoing pain or suffering.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The maximum severity limit on this licence will be moderate. Approximately 80% moderate and 20% mild severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In order to study the inflammatory response three components need to be examined; time, place and cell type. While place (organ) and cell type (leucocyte or stromal) can be examined relatively easily in humans, it is difficult and in some cases unethical to perform multiple biopsies and adoptive transfer experiments in human patients with chronic diseases such as Sjogren's Syndrome (SS) without underpinning preclinical data that support therapeutic utility. Furthermore, manipulating cells as a therapy has the potential to impact on the normal function of the individuals immune system. We will need to determine therefore, whether manipulating these cells as a therapy could affect an individuals ability to also fight infection. We therefore need to examine these diseases in models with a fully functional immune system analogous to humans. Small rodents are the lowest mammals that can be used to recapitulate the response of the human immune system to localised inflammation. Mice at a more immature life stage cannot be used due to their salivary glands not being fully developed. The model requires the use of live animals since our aim is to track changes in the immune response over a course of hours to weeks, therefore terminally anaesthetised animals cannot be used.

Which non-animal alternatives did you consider for use in this project?

We considered a range of co-culture models and organoid experiments as non-animal alternatives, using patient samples and archived tissue. These techniques will supplement where possible animal experimentation and therefore reduce the overall number of animal experiments needed.

Why were they not suitable?

We and our collaborators have pioneered a range of in vitro co-culture models that have furthered our understanding of leucocyte-stromal cell interaction in chronic inflammation. However we have now reached a point where we cannot proceed to test our ideas without resorting to animal models of inflammation. A key strength of our work is that it combines both human and animal models so that each can be used to inform the other and therefore minimise an over reliance on mouse models of disease. While there are no in vitro alternatives to this work, we will use cell culture experiments and target validation experiments on human samples alongside the program of work explained in this licence to support and validate our findings. We are confident that this approach will prevent us from developing a large program of work in vivo that is void of a functional relevance in human disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used specific mathematical calculations based upon previous studies and the likelihood of our interventions producing positive results to estimate the number of animals we will use in our study. For all experimentation, the lowest possible number of animals will be tested whilst ensuring that the experimental result is robust.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A key strength of our work is that it combines both human tissue work and animal models so that each can be used to inform the other and minimize an over reliance on mouse models of disease. This will give us the option to stop the line of research at any stage where our findings fail to show any significant increase in our understanding or therapy potential in Sjogren's Syndrome.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our previous work has mapped the specific time points in this model that are critical for the analysis of TLO development. This work will allow us to limit the number of observations and therefore animals required in each experiment, as we can now focus on specific time points depending on the objective of any specific experiment. To maximise the information gained from a single animal, we aim to perform multiple analyses on tissue obtained from each individual animal.

New interventions will first be tested for efficacy using in vitro models prior to use in vivo. Where new routes of administration or new interventions are being examined, pilot studies will first be established in 2-3 mice prior to full experiments. Subsequently these pilot data will be used in the specific mathematical calculations described above to ensure that we use the minimum number of animals needed to obtain statistically significant results.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Building upon previous work, we will use a well-defined and published mouse model of Sjorgrens Syndrome (a disease of salivary gland inflammation). We will induce salivary gland inflammation in mice by administration of a non-harmful virus into the submandibular salivary gland via a procedure that directly cannulates the salivary duct.

1. The procedures listed in this licence have been optimised to minimise discomfort for the animals. All cannulations are carried out under anaesthesia with analgesia provided both before and after the procedure, and as required over the days following virus administration. The virus administered has been altered to be non-harmful and does not result in widespread infection only local inflammation at the site of injection. Our experiments are short-term, taking place over hours or a few weeks, with very clearly defined humane endpoints. Mice that undergo procedures and/or mice with uncharacterised genetic mutations will be monitored closely and appropriate action taken if they demonstrate adverse effects. Animals will be humanely culled unless, in the opinion of the NVS or NACWO, suffering can be remedied promptly and successfully using no more than minor interventions, such as pain relief and hydration.

2. We have refined the protocol to immunise mice by single subcutaneous injection in the upper surface of the paw and not in the foot pad so as to cause less discomfort.

3. When using genetic mouse models we will, where possible, use inducible models where genes are switched on conditionally. This will limit the duration of any potential harm or suffering to the mice affected by a change in expression of that particular gene.

4. Where possible we will use established reagents and protocols that we have developed and refined to treat the mice. The lowest doses of agents that are well tolerated and effective will be used.

5. Where necessary, male mice transgenic for the gene of interest will be mated with WT females in order to exclude indirect effects on the progeny derived from gene overexpression in the pregnant female.

Why can’t you use animals that are less sentient?

Less sentient animals do not possess the same sort of salivary gland structure, and often their vascular tree and immune system do not fully represent that of humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have already heavily refined the cannulation protocol and the associated peri- and post-operative care. We will continue to seek advice from the NVS regarding best use of anaesthetics and analgesics, particularly for any mouse strains which have never been previously cannulated. Any strain which has never been cannulated will receive increased post-operative monitoring following the procedure. We will have to use injectable anaesthetic rather than inhalation anaesthetic due to needing access to the animals mouth. We are currently planning a pilot study in cooperation with the NVS on refining the procedure to use a shorter acting injectable which allows for faster post-operative recovery.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Animal welfare is a key consideration in all of our protocols, and we will be guided by our NACWOs and NVS in always ensuring that we are using best practice and the most refined techniques. All staff involved in animal experiments will review the literature on animal welfare. Following every experiment and regularly during group meetings we will review our procedures from a welfare standpoint to identify any potential for refinement.

If undertaking a systematic review, we will use SyRF (the free online platform for researchers) to perform a systematic review and meta-analysis of animal studies. This will allow us to keep up to date with any improvements in protocols and techniques which may reduce or replace the use of animals.

Finally, we will follow the LASA guidelines Guiding Principles for Preparing for and Undertaking Aseptic Surgery (www.lasa.co.uk/wp-content/uploads/2018/05/Aseptic-Surgery.pdf) and by the Home Office Minimum Standards for Aseptic Surgery (www.procedureswithcare.org.uk/ASMS2012.pdf) when undertaking aseptic surgery and providing analgesia. We will also follow the PREPARE and ARRIVE guidelines for our planning and reporting of our experimental findings.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I or a member of my research team will attend the 3Rs regional workshops that run at our establishment on a regular basis and sign up to the NC3Rs newsletter. We will also be reviewing the literature on regular basis in our journal clubs and through this network we will discuss any refinements that could be applied to our own work.

68. Determining Mechanisms of Tumour Development and Relapse Following Treatment

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Chemotherapy, lymphoma, neuroblastoma, drug resistance

Animal types	Life stages
Mice	adult, juvenile, pregnant, embryo, neonate, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to understand why cancer develops and how it changes in response to treatment, in particular, to determine why some cancers become resistant to therapy.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is one of the major causes of death worldwide with at least 1 in 2 people developing this disease in their lifetime and only half of these surviving. It is therefore clear that we must conduct more research to understand not only why cancer develops in the first place but also why treatment is not successful for many.

What outputs do you think you will see at the end of this project?

This research will lead to new information regarding the mechanisms of cancer development as well as the reasons for resistance to therapy. This work will be published in the scientific literature as well as presented to both lay and scientific/medical communities at research conferences and meetings. In particular, through interactions with clinical communities as well as pharmaceutical companies to enable the translation of the research findings into real clinical impact. This could be in the form of the development of new therapeutic targets and drugs and/or clinical assays that can be used to determine whether a patient will have a good outcome following treatment. Alternatively, this research may facilitate clinical decisions such as whether their treatment needs to be altered due to a lack of response to the therapeutic protocol.

Who or what will benefit from these outputs, and how?

In the short term, the outputs of this research will benefit those in the medical and scientific communities to facilitate future research activities. In the medium-term, we these findings will be applied to the design of clinical trials and associated biological studies to learn more about cancer in the real world, outside of the lab. In the long term, it is hoped to develop new treatment strategies that may include new drugs or different combinations of existing drugs to improve cancer outcomes.

How will you look to maximise the outputs of this work?

This research is part of many ongoing collaborative research activities, including Europe-wide and international research initiatives. In particular, both established research networks and clinical trials groups will be engaged whereby these data will be presented at network-wide meetings towards clinical application and to inform future research opportunities. In addition, via a Europe-wide training network, this research will also contribute to the training of fledgling research scientists and clinician scientists in order that this future generation will be better informed and will be able to take forward the findings of this research to real clinical application. The research conducted under this licence will also be applicable to patient groups globally including those residing in low-income countries that are affected by the cancers that will be studied. Important collaborations with such populations will enrich the work.

In all cases, the work conducted will be published in the scientific literature, disseminated to both lay and scientific audiences at meetings and conferences, and communicated to interested parties such as pharmaceutical organisations to initiate collaborative research activities.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our research uses mice to understand why cancer develops, in particular mice that have been genetically modified to express proteins suspected to cause cancer. This work is necessary as a mechanism to prove that certain changes to the DNA code drive cancer development. Furthermore, to determine whether inhibition of the activities of these proteins will be effective in the treatment of people with cancer. We also use mice that have no immune system so that we can grow human tumours in the lab. If the mice have an immune system, they will reject the human tumour. Adult mice with no immune system host human tumour cells and allow them to grow. We can try to cure them of the disease.

Typically, what will be done to an animal used in your project?

We will breed mice that have no immune system so that they do not reject the human tumours. However, an immune system is necessary to enable some drugs to work, in particular drugs called 'immunotherapeutics'. To study these drugs, we will develop 'humanised' mice. Humanised mice are mice that do not have their own immune system are instead injected with human blood cells to create a human immune system. Mice are reconstituted with human immune system cells (humanised mice) by intravenous (iv) injection. Tumour cells are grown under the skin (sub-cutaneous) of these mice so that tumour growth can be easily monitored by measurements with a type of ruler called a calliper, and by feeling the mouse at the site of injection. On occasion, the sub-cutaneous environment cannot support tumour growth and so we also attempt to culture the primary tumour cells at other sites in the body such as in the abdomen (intraperitoneal) or in the blood (intravenous).

Treatment of the mice with investigational drugs starts once the tumours reach a certain size (as determined by calliper measurements, or in case of intravenous/intraperitoneal growth, by looking for tumour cells in blood samples and using imaging techniques such as magnetic resonance imaging (MRI) scans). In order to better mimic the human scenario, whereby tumours are generally diagnosed when they are relatively large (preventing normal bodily activities leading to symptoms and subsequent diagnosis),

treatment commences when the tumours reach 0.5-1.0 cm at their widest point. Mice are humanely killed at the pre-defined study endpoint, which is either the end of the treatment regimen, or when the tumour reaches the maximum recommended size of 1.5 cm in any one dimension. In some cases, the tumours are allowed to reach 2 cm when more cells are required for downstream analysis (particularly when analysing tumour cell subsets such as the very small populations of cells that remain after therapy, so-called treatment persister cells, or cancer stem cells). In these cases, the mice for these studies are clearly identified before commencing the project and are monitored closely for side- effects of tumour growth (e.g., paralysis). Drugs are administered by the most appropriate route according to how drugs are metabolised by the body (so-called pharmacokinetic/pharmacodynamic parameters). This can be through the mouth, intraperitoneal (into the abdomen), sub-cutaneous (under the skin) or intravenous (in the blood) or via a mini pump implanted under the skin. In the past we have dosed mice with the drugs for a maximum of 30 days, but it has become apparent that this is not enough time – when the drug treatment has completed, despite it looking like the tumour has disappeared (even when using high resolution devices such as MRI scans), the tumour returns. As such, we would like to have more flexibility in drug delivery schedules allowing us to give the drug with a variety of treatment schedules (e.g., metronomic dosing (on, off, on, off drug etc), drug holidays (on, off for a long period of time, on etc.) and co-administration of different drugs over longer periods of time. In all cases, mice are closely monitored for any clinical signs that might indicate that the drugs are toxic, for example by monitoring the weight of the mice daily.

Genetically altered (GA) mice (mice that have had their DNA code altered) will largely be used for ex vivo (outside the body) studies of tumour cells. These mice express cancer-causing genes, so-called oncogenes, in certain parts of the body, and develop tumours at these sites. For the most part, the GA mice we use produce tumours of the immune system and therefore develop tumours at sites of immune system components such as the thymus (an organ that sits above the heart), spleen and lymph nodes. Tumours are detected by regular palpation (massage) of the mice, and we will incorporate imaging, such as MRI scans, at key timepoints and on presentation of clinical signs to allow early tumour detection to minimise suffering. To allow tumour growth, the GA mice are aged, sometimes as long as 15 months of age. The GA mice are also used to examine how tumours develop and, in these cases, the mice are killed by a humane method shortly after genotyping (checking the DNA code) for analysis of tumour cells outside of the body. We may breed GA mice to incorporate other genetic elements into our studies. For example, clonally expressed T cell receptors (e.g., TCR transgenic mice), the 'flags' on the surface of immune cells that recognise invading pathogens, to see if these also contribute to tumour growth.

To assess the effects of potential secondary events on tumour development, i.e., environmental factors that might contribute to the development of cancer, GA mice might also be administered stimulatory agents to assess the rate of tumour development and incidence in comparison to control groups. For example, TCR transgenic mice expressing

oncogenes in immune cells might be dosed with agents that activate the immune cells. This might include ovalbumin protein or components of this, called peptides. We will also examine the role of other environmental agents such as microplastics that are shed from a range of plastics and polymers. These agents may be delivered by the routes previously described for drug administration but might also include delivery through the nose, perhaps in an aerosol.

Typically, work conducted under this licence allows mice to live under standard conditions until tumours develop. As soon as tumours are detected, the mice are humanely killed, and the tumours harvested for studies conducted in the lab and outside of the body (ex vivo).These tumours do not grow without a host biological system and the mouse provides the least sentient being that can facilitate tumour growth. However, at all times we attempt to grow the tumour cells in the lab in plasticware and as technologies evolve that facilitate this, we will incorporate this more into our research approach.

In the case of human tumours grown in mice with no immune system, the tumour cells are typically injected and grown under the skin on the hind flanks of the mouse. In some cases, for example if we are studying leukaemias, cancers of the blood, the cells may be injected into a blood vessel or at another site that is more representative of where the tumours grow in humans, such as the abdominal cavity. The mice are monitored continuously for their well-being, and should the tumours prevent their normal activities such as feeding and moving, the mouse is humanely killed, and the tumour harvested for ex vivo studies or re injection into a new host mouse. In some cases, we treat the mouse bearing the tumour with different drugs to determine if these are good options to cure the tumour and prevent its continued growth. In these cases, the mice are again closely monitored for any clinical signs, not just those that might be caused by the physical presence of the tumour, but also those that might be due to the drug being administered. If any of the mice show clinical signs, they are immediately humanely killed. However, this is a rare occurrence as we monitor tumour growth not just by eye, but also using imaging modalities such as MRI scans and ultrasound so that tumours are harvested before they impact the wellbeing of the mouse. Drug treatment experiments are typically conducted over periods of months with the initial months being the period in which the tumour grows and in the subsequent time, the mouse is being treated. If the treatment approach is not successful and the tumour continues to grow, the mouse is humanely killed before the tumour reaches a size that would impact on the wellbeing of the mouse. On some occasions, when we think the mouse has been cured as we cannot detect any tumour cells in the body, the mouse is allowed to survive in case the tumour relapses or returns, so that we can study the tumour cells at this point to understand why they have grown back. To prove that the cells which grow back are truly resistant to the drugs, we may also treat the mice again with drugs to see how the tumour responds.

What are the expected impacts and/or adverse effects for the animals during your project?

In general, the mice do not experience any adverse effects from tumour growth as we carefully monitor their health status. We do not allow tumours to grow in size to the extent that they affect the wellbeing of the mouse. However, should the tumours affect the wellbeing of the mouse, it is normally due to growth of the tumour near to the hind limbs which can cause paralysis. Affected mice are humanely killed immediately. As the mice are checked daily, at most, a mouse would experience paralysis for no more than 24 hours.

Some mice can experience weight loss due to the effects of the therapy, as do people with cancer undergoing treatment. However, whilst on the protocol, the mice are weighed daily to observe any changes that might trigger humane killing such as weight loss and lethargy. Should a mouse show any clinical signs, they are immediately humanely killed.

On rare occasions, a new drug being tested can have unexpected adverse effects. However, to minimise this possibility, we search the literature for existing and known toxicity data for a given drug before use. If such data does not exist, we start experiments with low doses of drug in one or two mice as a pilot project and scale up dependent on the results. As the mice are checked daily, at most, a mouse would experience paralysis for no more than 24 hours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mouse: moderate 20%
- Mouse: Mild 80%

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cancer is a complex disease that requires a number of host factors to be 'just right' for growth and propagation. For the most part, as a scientific community we do not know what these factors are, but they likely involve food and nutrients provided through the lymphatic and blood circulation as well as locally provided compounds such as growth factors and

messengers produced by the immune system and the cells providing the structural support of the tumour. Until the conditions required for tumour growth can be emulated in vitro, we need to continue to use the mouse to host tumour growth and to be able to examine the tumour as essentially, a new organ. Secondly, how a tumour responds to treatment can vary depending on how the body as a whole reacts to administration of a drug, specifically, how the body processes the drug. Drugs are normally given to a patient orally or via the blood stream - these routes of administration can affect how a drug is metabolised and therefore its efficacy in the body. Therefore, to fully assess the activity of any given drug in affecting tumour growth, in vivo studies are ultimately required.

Which non-animal alternatives did you consider for use in this project?

In vitro growth of tumour cells as single layers of cells or as organoids.

In vitro drug screens on established cancer cell lines.

Why were they not suitable?

Our experience shows that the tumour cells do not grow in these in vitro systems. It is clear that they require the environment of the mouse host to survive, likely due to the requirement for some, as yet unidentified growth factors.

For drug studies, whilst established cancer cell lines can be informative in determining the efficacy of a drug, the effects of bodily systems on the metabolism and, in some cases, activation of the drugs, must be determined in vivo. In addition, the established cell lines have evolved in the lab to become less representative of the tumours that were first removed from the patient to establish these cell lines, i.e., the cell lines have evolved and adapted to growth in a petri dish and no longer truly represent the genetics of the original tumour.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In the previous project licence, we used 800 mice in the first 4 years of the project. I anticipate that we will continue to use mice at this rate as this current application is a continuation of the prior work.

However, in the last project period, our research was inhibited by the pandemic, and we had not secured sufficient funding to complete all of the proposed work. We now have

secured considerable research funding for all aspects of the research proposed and do not anticipate interruptions due to global pandemics. As such, I expect that are usage will triple over the 5 years of the project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We follow PREPARE guidelines in our experiments. In particular, in the design of drug studies, we perform Power calculations to make sure we use the minimum number of mice to reach significant results and are assisted in this with the use of the NC3Rs experimental design assistant.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have developed in vitro methods for tumour cell growth using feeder cell lines to conduct drug screens with patient-derived material. Where cells cannot be grown in vitro, we perform experiments with primary cells ex vivo. Ultimately, drug studies have to be conducted in vivo, particularly when in vivo drug activation is necessary.

We have also begun to use imaging techniques in our studies so that tumour development in vivo can be monitored more easily. In particular, we have used MRI to assess tumour growth and will apply this technique more in the future. We will also use ultrasound in this manner.

If a drug has been tested before in mice for the purposes we intend, we search the literature first for previously conducted toxicity studies, if available, to identify LD50 doses (the dose that has been shown to lead to the death of 50% of the animals in that study). We then conduct control experiments starting with low doses and incrementally increasing them whilst monitoring mice for any clinical signs before progressing to each higher dose. These pilot studies allow us to assess the tolerability of the drug and the amount of drug required to kill the tumour cells.

If a drug has been tested in vivo before, we assess the literature to decide on the best drug dose for our studies rather than repeating efficacy studies.

Mice are randomly assigned into treatment groups and are blindly administered drugs, the identity of which are unknown to the administering technician.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice:

NSG immunodeficient mice

Transgenic mice including strains expressing oncogenes predisposed to developing cancer

Techniques:

Sub-cutaneous, intraperitoneal or intravenous injections of tumour cells: these routes of tumour delivery are conducted by fully trained personnel without anaesthesia and have very minor and transient effects on the mouse. The mice are closely monitored after the procedure for any clinical signs.

Intraperitoneal, intravenous, oral and sub-cutaneous mini-pump delivery of drugs: these routes of drug delivery have transient and mild effects on the mice when conducted by trained personnel. They are conducted without anaesthesia but by fully trained individuals. Surgery to implant sub-cutaneous mini pumps is conducted under sedation or brief general anaesthesia from which the mouse is monitored closely for a full recovery. The mini pumps, when inserted correctly, have no effect on the wellbeing and normal activity of the mouse.

Imaging - MRI, ultrasound and PET/CT: allows us to monitor tumour growth when they are not detectable by palpation alone, preventing 'unseen' tumour growth to continue to the point where clinical signs ensue. Mice undergo sedation to improve their comfort during the procedure.

Why can’t you use animals that are less sentient?

In order to study the development of cancer it is important that we consider the roles of the whole body in order to better model the scenario in humans. This requires components of the extracellular matrix, stromal cells, immune system components and a blood supply, factors that at present are not possible to fully mimic in vitro. In addition, a defining feature of cancer is the ability to invade surrounding tissues and to metastasise - these can only be truly modelled in vivo. As such our research to investigate origins and development of tumours will continue to be performed in vivo with GA mice. We will also continue to use mice as we have a plethora of GA mice that have previously been developed and characterised available to us. In order to conduct experiments assessing established tumours, we employ patient derived xenografts (PDX) as the least sentient system in which to grow primary human- derived tumours. With our PDX models we attempt to grow the tumour cells in vitro after each passage through the mouse but have had limited

success even with 3D culture conditions. We continue to refine these in an attempt to establish non-animal alternatives and reattempt in vitro culture at each stage.

When short term culture is sufficient, e.g., for determination of early chemotherapeutic sensitivity, we conduct initial experiments in vitro to determine active drug concentrations. However, due to effects of bioavailability, experiments are then conducted in vivo to predict the potential consequences in human patients.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will add enrichment to the home cage (such as tunnels) and conduct non-aversive handling (tunnel and cupping), habituation and acclimatisation, e.g., if mice ordered in from an external supplier, will be allowed a habituation period of at least 1 week so the mice can settle into their new environment.

When performing dosing, mice are first acclimatised to the handling technique leading up to the day of dosing, (such as scruffing), so as not to overwhelm the animal all at once.

We will employ immunodeficient mice to propagate human tumours and wild type congenic mice to propagate tumours derived from GA mice. Both models will be used to assess the impact of chemotherapeutic treatments on tumour response and clonal tumour evolution. The most appropriate site for tumour propagation in vivo will be determined based on experience and will largely constitute sub-cutaneous growth of solid malignancies and intraperitoneal or intravenous for liquid tumours. All of the techniques we use are already well established and result in minimal suffering to the mice. We perform all sub-cutaneous injections with two trained animal researchers so that the procedure is quick and accurate (one to hold the mouse, the other to inject). We also capture mice using non-tail handling techniques prior to scruffing for injection (the NSG mice are very placid and respond well to handling). All trained animal researchers are also regularly assessed in their use of techniques by experienced animal technicians in the unit. We also attempt, when possible, to employ the services of animal technicians within the unit who regularly perform these techniques and are therefore very experienced resulting in minimal suffering to the mice. When performing injections, we suspend the reagents in the smallest volume possible to reduce the time taken to undertake the procedure although this is dependent on the solubility of the compound. Tumour growth is monitored by calliper measurement or palpation of internal tumours and liquid tumours by blood sampling. When attempting to grow a new tumour for the first time, the mice are carefully monitored using a staging system for clinical signs until we can establish the growth properties of that tumour. We also employ imaging techniques such as MRI and ultrasound where possible to monitor tumour growth. In this manner, tumours can be monitored, and the mice culled before overt clinical signs manifest.

Our mice are expected to develop tumours and therefore in order to minimise suffering we use an in- house designed Excel database to record experiments and severity levels

reached. We also check our mice for tumour development by palpation and observation of clinical signs on a daily basis. Sub- cutaneous tumours, once established, are measured using callipers on a daily basis to ensure the size limits are not exceeded. At the same time, the mice are monitored for any clinical signs and any issues with the grafted tumour; in particular, tumours are monitored for ulceration, any growth that limits movement and general condition. Mice experiencing any issues with grafted tumour humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Advice on experimental design will be sought from the statistical services unit and by using the NC3rs experimental design assistant (www.nc3rs.org.uk/experimental-design-assistant-eda). Factorial design of experiments will be considered where necessary to minimise animal usage and for quantitative experiments, power calculations will be used to determine sample sizes generally using a significance level of 5%, a power of 80% and a least practicable difference between groups of 25%. However, we will also plan experiments based on our prior experience of working with the described model systems and dependent on experimental limitations, for example, the number of cells that can be isolated from a human tumour and therefore propagated in the mouse; we expect treatment group sizes to consist of between 6-8 mice, but this may be less. Each experiment will be conducted following the development of a study plan whereby a statement of the objectives of the experiment, a description of the experiment, covering such matters as drug dose, frequency of dosing, number of mice per group, experimental endpoint, data analysis and the experimental material are described. Mice will be randomly assigned to treatment groups and attempts will be made to blind studies. In essence, we will conduct all experiments according to the ARRIVE guidelines in accordance with NC3rs procedures in order that data can be disseminated by publication in the peer reviewed scientific literature. We will also consult the 'Guidelines for the welfare and use of animals in cancer research' published by Workman et al., British Journal of Cancer, 2010.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly monitor the scientific literature for advances in the modelling of human cancer, in particular paying attention to advances in the development of in vitro organoids and 3D bioprinting and will review journals such as ATLA (Alternatives to Laboratory Animals) Journal: https://journals.sagepub.com/home/atla. As these techniques improve and are better able to mimic the human tumour microenvironment, we will apply for funding so that we can adapt our research to these technologies. We will also regularly refer to the LASA Guidelines: https://www.lasa.co.uk/current_publications/ and to the RSPCA website (http://science.rspca.org.uk/sciencegroup/researchanimals).

We also subscribe to the local 3Rs email enquiry list that provides regular updates on advances in the 3Rs and their implementation and signposts the reader to important and

relevant resources. We also regularly consult the local 3Rs search tool. We also subscribe to the NC3Rs webinar series and will attend these events as appropriate as well as consult the NC3Rs Gateway (https://f1000research.com/nc3rs).

69. Establishment of Patient Derived Xenografts from Biopsy Samples

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Xenograft, Patient biopsy, Tumour Microenvironment, Biobank

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to establish Patient-Derived Xenografts (PDX-man-to-mouse tissue graft)from a variety of human tumour biopsy samples and to identify those elements of the environment in which the tumour grows (Tumour Microenvironment-TME) that are critical to ensuring that mouse xenograft tumour models optimally represent the human disease. As part of this process, we aim to generate sufficient tissue for use in laboratory-based drug development assays or other projects and to freeze and store samples in a Biobank.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer directly affects 1 in 2 of the population during their lifetimes and despite significant progress being made in the last two decades, it still remains a major cause of mortality meaning improved therapies are required.

Despite advances in transgenic (genetically modified) technology, use of new species such as zebrafish and the availability of new strains of rodents, there still remains a level of disconnect between laboratory evidence of effectiveness and toxicity of new anti-cancer agents and the outcome of their trials in the clinical setting with drugs tested. Thus, there is currently a drive to determine ways of ensuring animal and laboratory models of cancer are more predictive and a number of areas are being focused on including:

- Use of ‘close-to-patient’ tissue
- Humanisation of the Tumour Microenvironment (TME)

Therefore, the main purpose of this licence will be to establish xenografts derived from fresh patient samples, incorporating elements of the TME if appropriate, to provide close-to-patient tissue to use in our experimental animal and laboratory models, which we hope will be better able to represent the variety between and within patients and will, consequently, be more clinically relevant.

Given the variety of cancers which affect the population, we will investigate a number of different tumour types, namely

Colorectal Gastric Pancreatic

Oesophageal Brain

Lung Cholangiocarcinama Prostate

Ovarian Breast

What outputs do you think you will see at the end of this project?

These finding will directly feed into both our cell based and experimental animal based projects, by enabling us to produce more accurate "human-like" models, supporting cancer drug discovery pipelines.

Data and findings generated under this project will be published in peer reviewed journals and disseminated at national and international meetings by several members of the group.

The establishment of a Biobank of frozen tissue will provide a valuable resource for future studies.

Who or what will benefit from these outputs, and how?

The main beneficiaries will be scientists working on developing clinically-translatable drugs and imaging agents who will use the models in their research. In the longer term the beneficiaries will be patients as the models will enable development of more clinically-effective, affordable drugs and imaging agents which will improve cancer diagnosis, treatment and outcomes.

During the lifetime of this project, this will arise from the generation of superior ‘humanised’ cancer xenograft models incorporating human cell components, derived from patient tissue, which are more representative of the patient’s tumour in terms of biology and response to treatment than standard xenograft models. They can also be used to establish flat (2D) and 3-Dimensional laboratory-based model systems in order to reduce/replace animal use during the early stages of drug discovery. These would provide better, more robust models that are more predictive of individual patient response, and will therefore, provide more accurate results in drug development research which can be used both by ourselves and our collaborators. This may result in the development of more effective drugs that do not fail when they reach the clinical phases of drug development, reducing the numbers of animals used in developing drugs that are unlikely to be clinically useful and the costs and time associated with this process. Hence, ultimately this will also benefit patients by reducing disease burden and mortality through development of better drugs.

Longer term, through the establishment of a bank of frozen PDX samples, we will secure and develop a valuable resource for future use by, not only us, but also shared with other collaborators whose projects would also benefit from this technology, which, in turn, will reduce the overall numbers of animals needed to generate tissue for their experiments.

We also aim to be able to capitalise on knowledge gained during the above processes to further develop our cell based models and develop methods to produce "close-to-patient" cells in the laboratory, reducing/replacing the use of animals for this purpose too.

How will you look to maximise the outputs of this work?

We will maximise these outputs by publishing findings in high impact, open access peer reviewed journals and by presentation at national and international meetings. We will utilise existing networks e.g. BACR, EACR, NC3Rs, LASA and other national and international working groups to further disseminate our findings and we expect both positive and negative results generated will be made available.

The establishment of a Biobank of frozen tissue will provide a valuable resource for future studies and, where possible we will share this resource, and our knowledge and technical skills with other University and external researchers to fully exploit the impact of our research.

We will also publicise our findings via Patient Participation & Involvement groups, as appropriate.

Species and numbers of animals expected to be used

- Mice: 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The application of our experimental technologies requires a species with a body structure similar to a human, and it must also be able to generate a sufficient amount of tissue for our experimental needs. Mice are the lowest species in which a genetic mutation has produced an immunodeficiency (a full or partial impairment of the immune system) which allows the growth of human tumours without rejection. There are a number of different strains available with varying degrees of immunodeficiency so the specific strain choice is dependent on the level of immunodeficiency required to allow growth of the particular tumour type being implanted.

Experience has shown us which immunodeficient mouse strain is most appropriate for particular tumour types, but we will always try to improve take-rates by trying new strains as they become available. Therefore, pilot studies may be carried out to identify the most suitable immunodeficient species for a particular tumour type.

Adult mice are the most appropriate age as our samples come from adult humans.

Typically, what will be done to an animal used in your project?

All mice will undergo at least one procedure i.e. it will have a small amount of tumour (fragments or cells) taken from a patient biopsy sample implanted under the loose skin on its flank or, in the case of breast tumours, into the fat pad under the mammary gland, using an appropriately sized needle. Local anaesthetic may be rubbed on the skin first if a large sized needle is necessary. The tumour fragments may be mixed with other cell types and/or a biological support gel to stimulate or encourage growth.

The tumour, which stays as a discrete lump under the skin, will be measured at least weekly, depending on growth rate, until the maximum allowable size (1.2cm diameter) is reached, at which point the animal will be humanely killed and the tumour removed for processing.

Hormone supplements may be required to stimulate certain tumours to grow i.e. oestrogen for breast and ovarian tumours, and testosterone for prostate tumours. These supplements will be delivered via the feed or the drinking water for the duration of the experiment.

In 20-25% of mice which have undergone the tumour implantation procedure, other cell types making up part of the tumour's natural environment (TME) may be injected into or around the developing tumour no more than 3 times during the experiment. These mice will, therefore, undergo between 2 and 4 procedures.

Growth rate of the tumour depends on which type it is, but most experiments end after 3 months, with around 20% lasting up to 6 months.

What are the expected impacts and/or adverse effects for the animals during your project?

In a typical case (95%), the mouse will experience minimal discomfort from the implantation of tumour fragments via an implant trochar (large gauge needle) under local anaesthetic and physical restraint. The resulting tumour growth will be monitored by measuring with callipers, again while physically restrained by an experienced technician. The growth of the tumour should have no impact on the animal’s health and welfare and the animal will be killed when the tumour reaches the maximum allowable size (1.2cm mean diameter). Injected tumours grown subcutaneously or in the mammary fat pad, may ulcerate or develop shiny patches which could lead to ulceration, independent of tumour size, in around 5% of the animals. The animals will, therefore, be observed regularly as soon as growth is established, and any mouse will be humanely killed and the tumour excised as soon as the skin covering the tumour appears to be stretched or broken.

Due to their position, mammary fat pad tumours may interfere with normal movement of the hind limb (5%). At the first indication of this, the animal will be killed, regardless of tumour size.

Injections of cells into or around the tumour should not cause any side effects. However, if any damage to the skin, such as ulceration, is seen as a result of these injections, the animal will be humanely killed.

There may be specific adverse effects to any hormone substances being delivered. Therefore, animals receiving such substances will be carefully monitored and any exhibiting mild symptoms, such as minor weight loss or signs of dehydration or urine scald, will revert back to normal diet or water until recovery is observed. Furthermore, testosterone, may cause increased aggression and fighting amongst males; animals will be separated and singly housed where appropriate.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All mice will experience no more than mild severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

While our experimental programme is focused strongly on in vitro (laboratory-based) non-animal modelling, it is currently not possible to generate a sufficient amount of cells required to develop these models directly in petri dishes from patient samples. Therefore, mice are still required as a vector for this initial tissue establishment and maintenance, until sufficient stock tissue has been generated for in vitro experimentation and investigation, and for banking down by freezing, when the line will be closed in the mice.

Which non-animal alternatives did you consider for use in this project?

A variety of cell culture techniques, including single layer (2D) and 3-Dimensional growth methodologies were considered.

Why were they not suitable?

These other methods currently do not generate a sufficient volume of tissue for the subsequent in vitro

studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used per sample is dependent on the amount and quality of tissue supplied from the operating theatre. Typically, after preparation, the tissue will be

implanted into 1 or 2 mice, but never more than 3. Once the initial samples have grown they will be transplanted into the minimum number required for tissue generation and examination by e.g. histology, immunohistochemistry.

Typically, this will be a further 2 animals but exceptionally, may be up to 4 depending on the amount of tissue required, and how much good quality tissue is available for transplant. This will happen for as few sequential transplants (passages) as possible to generate sufficient tissue to fulfil the above objectives, typically 5 times, although some tumours may stop growing after e.g. 2 passages, or not grow at all. When sufficient stocks have been generated and banked down, or when growth stalls, the line will be closed and will no longer be passaged on.

By using disaggregated (separated out) cells rather than tumour fragments, where the sample is suitable, due to the number of cells generated using this protocol, we are able to implant more mice per tumour thus reducing the number of donor mice required.

Therefore, group size is sample size driven to safeguard the quality and quantity of the developing tumour xenograft, rather than to provide statistical significance, so we will use the minimum number of animals needed for the amount of tissue required, based on experience and data gathered from previous licences.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

From previous experience we calculated our total number required based on the minimum number of animals necessary for the tumour sample size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will introduce refinement methodologies developed during our previous PPL to optimise quality and quantity of tissue generated from each sample, thereby requiring fewer animals to produce the required amount of usable tissue. For example, we have shown that the take-rate is higher when human stromal cells are co-implanted or, for particular tumour types, in specific immunodeficient mouse strains. Thus, we have fewer numbers of mice in which no PDX is regenerated as a result of refinements we have introduced.

We will also carry out pilot studies on new mouse strains to see if they can produce better growth and, therefore, require fewer mice to generate sufficient tissue, and incorporate these strains into the project if they prove to be successful.

This study also involves the generation of a frozen tissue biobank which researchers will be able to use in further studies reducing the need for more animals to be used and maximising the tissue use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The application of our experimental technologies requires a species with an appropriate mammalian body system, and it must also be able to generate a sufficient amount of tissue. Mice are the lowest species in which a genetic mutation has produced an immunodeficient status which allows growth of human tumours without rejection in a clinically relevant setting. The strain choice is dependent on the level of immunodeficiency required to allow tumour growth as the genetic background may be a factor in determining tumour establishment.

Experience has shown us which immunodeficient mouse strain is most appropriate for particular tumour types, but we will always try to improve establishment and growth rates. Therefore, pilot studies may be carried out to identify the most suitable immunodeficient strains for a particular tumour type, and to try new strains which may become available.

All mice will be housed in containment systems (isolators or IVCs), with appropriate barrier husbandry procedures, in order to protect the mice from possible infections due to their weakened immune status.

Our procedures have been refined to minimise suffering by using local anaesthesia e.g. EMLA cream, and a trochar (large gauge needle) implant method rather than a surgical intervention as the impact of induction and maintenance of general anaesthesia would be greater than that of this method. The anaesthetic cream provides sufficient anaesthesia during recovery from the trochar introduction and further anaesthesia or analgesia is not required during the tumour growth phase as the scientific end point is reached prior to the tumour causing any pain or discomfort to the animal.

Implantation of disaggregated cells is even less invasive than the trochar method, being performed as a subcutaneous (under the skin) injection with a needle rather than a larger implant trochar, however, not all samples are suitable for this methodology. This methodology also creates greater consistency of growth between animals.

In the case of hormone dependent tumours e.g. breast and prostate, we deliver the necessary hormones by incorporating them into the diet or water, rather than by pellets implanted under the skin, having worked closely with the diet company to establish an

appropriate dose equivalent to that of the subcutaneous pellets. This refinement is less invasive, as it is not a surgical procedure, and will reduce the commonly seen effects of oestrogen pellets such as urine scald or bladder stones.

The favoured site for tumour implantation will be subcutaneous, under the loose skin of the flank, with the exception of breast tumours which will be implanted into the fat pad containing the mammary glands (also via the subcutaneous route) as these sites will not compromise the animal's movement or normal behaviour and are easily accessible for measuring.

Primary tissue, particularly oesophageal, gastric and colorectal tumours, may contain residual bacterial contamination. To prevent infection, the primary tissue is washed and incubated with antibiotic media overnight prior to implantation.

Other biological supporting components, such as stem cells, are administered by the injection routes which deliver them directly to the tumour, and these routes should cause no more than transient discomfort.

The mouse's weight will naturally fluctuate during the course of the tumour growth but, given the minimal impact of the entire procedure, it should not fall by below 10%. Therefore, this would be the point where we would take appropriate action, rather than the more generally accepted 20%.

Why can't you use animals that are less sentient?

Adult mammals are required to replicate the body systems and life stage of the human patients donating samples, thereby ensuring appropriate comparison and relevance to human body systems. Mice are the lowest sentient species in which tumours will grow in this comparative manner.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be monitored by trained technical staff (the same ones who have carried out the procedure and, therefore, understand the model) daily and they be weighed and tumours will be measured at least weekly. The maximum allowable tumour size will be as detailed in the NCRI Guidelines i.e. a mean diameter of 1.2 cm. However, any loss of condition or mobility or reddening/thinning of skin which will lead to ulceration will lead to the animal being humanely killed, regardless of tumour size.

All procedures will be regularly reviewed to see if any further refinements can be introduced.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The project will be carried out under the following guidelines-

NCRI Guidelines for the Welfare and Use of Animals in Cancer Research LASA Best Practice Guidelines

Norecopa PREPARE Guidelines

Findings will be reported according to the NC3Rs ARRIVE Guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will have regular liaison with the Named Persons and the AWERB.

I will keep up to date with publications and bulletins from bodies such as NC3Rs, RSPCA, Laboratory Animals.

My membership of Animal Science and Welfare bodies and attendance at their meetings and workshops and my network of colleagues working in similar fields will also keep me abreast of latest development, and I will take every opportunity to introduce any suitable refinements into the protocol, which I will then disseminate further via the above avenues.

70. Enhancing Neuroprotection through Activation of ERK5 MAPK Signalling

Project duration

2 years 0 months

Project purpose

(a) Basic research

(b) Translational or applied research with one of the following aims:

(i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Neurodegeneration, Neuroprotection, Alzheimer’s disease, ERK5, MAPK

Animal types	Life stages
Mice	neonate, adult, pregnant, embryo, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The ERK5 MAPK pathway communicates signals from the surface of the cell to the DNA in the cell nucleus and is known to support the survival of some cell types. The aim of this project is to investigate the role of the ERK5 MAPK pathway in neuroprotection by determining if activating the ERK5 pathway can protect neurons from damage typically observed in the early stages of Alzheimer’s disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The primary outcome of this project is to establish that activation of the ERK5 MAPK pathway is neuroprotective. As well as furthering our understanding of the ERK5 pathway in providing neuroprotection in Alzheimer's and other neurodegenerative diseases, this work could also identify new treatment targets.

What outputs do you think you will see at the end of this project?

The key output of this project will be the confirmation or rejection of the hypothesis that the ERK5 MAPK pathway protects neurons from damage typical in early Alzheimer's disease. The results from this proof-of-principle project will then inform further studies that will validate the ERK5 MAPK signalling pathway as a drug target in an Alzheimer's disease model such as APP/PS1 (GA) mice.

Who or what will benefit from these outputs, and how?

Answering the key research question whether activating the ERK5 pathway is neuroprotective will be beneficial for basic science as well as for the advancement of therapeutic treatments. In the shorter term, this project will lay the foundation for future work aimed at testing the mechanisms involved as well as validating ERK5 as a novel treatment target in an Alzheimer's disease model. The long-term aim of this work is to identify and characterize novel therapeutic targets and to developed them for the treatment of neurodegenerative disorders.

How will you look to maximise the outputs of this work?

All findings from this and any follow-on projects will be published under consideration of the ARRIVE guidelines. New knowledge, such as confirmation of the neuroprotective properties of the ERK5 MAPK pathway, will be further disseminated at scientific conferences (e.g. at Alzheimer's Research UK meetings) and through scientific seminars. For projects following on from this pilot study, we will build on existing collaborations with experts in electrophysiological measurements of synaptic plasticity and behavioural paradigms to maximise the impact of the work. This will allow the team to address the aims from different angles, thus maximising the information that can be gained from the animal models.

Species and numbers of animals expected to be used

- Mice: 190

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Alzheimer's Disease is a very complex condition that affects neuronal morphology and function. To date, no established cell line provides the level of synaptic complexity and neuronal functionality required for this project. It is therefore necessary to collect and grow neuron cell cultures from young mice. Mice are the established and well-documented source for the preparation of primary neurons, specifically from the hippocampus region of the brain. The project utilizes well-established protocols, which will ensure the greatest chances of successful cell growth, thus reducing the number of required animals.

For future studies, the genetically modified mouse model from this project offers the additional opportunity to further study the ERK5 signalling pathway in an Alzheimer's Disease model by studying their behaviour and taking electrophysiological recordings of synaptic plasticity.

Typically, what will be done to an animal used in your project?

Animals with a genetic alteration than is not expected to cause adverse effects will be bred by natural mating. The animals will have a sample taken (usually an ear biopsy) for genetic analysis. They will be killed, either by a schedule one method, or by decapitation (young mice only) if fresh brain tissue is required.

What are the expected impacts and/or adverse effects for the animals during your project?

No adverse effects are expected for live animals, the transgene will only be induced in the cell cultures.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild – All animals

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The proposed experiments have to be carried out in neurons, as the mechanisms involved in the modulation of synaptic processes cannot be investigated in any other cell type. Mice are the established and well-documented source for the preparation of primary neurons, specifically from the hippocampus. Lower vertebrates or invertebrates do not have the complexity of the neuronal networks required for this study of neurodegeneration.

Which non-animal alternatives did you consider for use in this project?

Established cell lines.

Ex-vivo wildtype (non-GA) primary cells. UK Brain bank.

Computer modelling.

Why were they not suitable?

To date, no established cell line can substitute for the level of synaptic complexity and functionality required for this project. In vitro assays cannot adequately model the complete array of molecular and cellular interactions necessary to fully understand the processes in the brain.

Ex-vivo experiments using primary cells from wildtype animals are useful for certain studies. However, all currently available in vitro methods to genetically modulate the pathway (such as transfection or transduction with viruses) will only reach a small sub-population of the primary cells. Genetic models such as the one proposed here offer the significant advantage that all of the cells will be equally modified, which is essential for obtaining reliable data for this project.

The UK Brain bank offers a range of human brain tissue samples, which will be considered for follow- on projects. However, the current project requires live cells.

Alternative methods such as computer modelling are not yet advanced and accurate enough to predict the effect of genetic knock-in and would not allow us to achieve the objectives of this project.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The key consideration for estimating the numbers of animals was the number of offspring required to produce the primary neurons necessary to achieve the objectives of the

project. The calculations have been based on similar experiments previously performed in my laboratory.

5 breeder pairs (10 animals) are needed to produce the required pups within the time frame of the project. Finally, the caMEK5DD mice will be received as heterozygous animals that need to be bred to obtain the homozygous mice required for the experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The breeding strategy has been designed following advice from the JAX website and discussions with the BSU staff. The breeding scheme to produce animals for the preparation of primary cells has been devised such that equal numbers of GA and control animals will be produced, so that there will be no surplus animals at this step. Statistical power calculations have been used to estimate the number of animals required to obtain statistically meaningful (significantly different) results. Finally, the use of GA animals will enable the activation of the ERK5 MAPK pathway in all primary cells obtained, increasing the statistical power of the experiments and thus reducing the number of animals from which cells will need to be prepared.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding has been designed to produce the required genotypes in a 50% ratio. Experiments will be conducted on both male and female animals, as the gender is not relevant for the ex-vivo experiments in primary neurons. All animals that will be killed (including the breeder animals at the end of the project) will be offered for use to other researchers within the university to make use of tissue from the culled animals. Throughout the project, all experiments will be analysed by appropriate statistical methods (ANOVA followed by a post-hoc test for pairwise comparisons). The experiments for the relevant experimental group will be stopped when statistical significance at $p<0.05$ has been reached, even if less than predicted numbers of experimental repeats have been used. The experiments will also be stopped if it becomes evident that it is likely that a specific treatment will not result in a statistically significant difference. Advice will be sought from biostatisticians if required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are universally used for work involving genetic alterations and they are the established animals for the preparation of primary neurons. This project is designed such that the transgene will only be expressed ex-vivo, i.e. in the established primary cell cultures. This ensures that there will be no adverse phenotypes present in the mice resulting from the transgene, thus minimizing any detrimental effects.

Why can’t you use animals that are less sentient?

The breeding of the GA mice has to be performed with adult mice. However, as the transgene will not be expressed in these mice, no adverse phenotypes are expected to result from the procedure. The primary cells will be prepared from neonatal mice, which is the optimal time for obtaining viable neurons. Neurons from less sentient animals will not have the required complexity for this project.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

There are no expected harmful phenotypes resulting from the breeding of the GA mice as the transgene will only be activated after killing. To monitor for any unexpected harm, the animals will be regularly monitored. Animals are checked daily (twice on weekdays) by competent animal care staff who will notify the PIL holder and/or NACWO of any indication of pain, suffering or distress including but not limited to abnormalities in behaviour and/or appearance, such as poor coat condition, piloerection, unusual posture (hunching), discharge or lack of grooming to eyes, ears, nose, ano- genital region, facial grimace, obvious signs of injury, abnormal movement, reduced activity, reluctance to socialise, reduced alertness, vocalisation, indications of reduced eating or drinking. We do not expect to see any harmful phenotypes in relation to the genotype, but litter sizes and health will be monitored for any indication of a decline in health status. Breeder animals will not be kept longer than necessary – up to 1 year - to prevent age-related adverse effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The key procedure for this project is the breeding of GA mice, which will be using standard protocols as provided by ASRU. The “Breeding and colony management - Best practice guidance” on the NC3R website will also be used to ensure the most refined way to breed the animals. In addition, the ARRIVE Guidelines 2.0 will be followed as best practice for reporting the outcome of all experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The investigator and researcher working on the project will maintain regular discussions with the NACWO and animal technicians to review current approaches and stay up-to-date with any new 3R developments. The BSU User Group also shares relevant information including NC3Rs and Norecopa newsletters.

71. Trypanosomiasis: Pathogenesis, Diagnosis and Treatment

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Trypanosome, pathogenesis, virulence, blood-brain barrier, neuropathology

Animal types	Life stages
Mice	adult, juvenile, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Trypanosomiasis is a parasitic infection affecting both humans and animals. It affects some of the poorest regions in sub-Saharan Africa. The disease can be fatal to both humans and domestic animals causing significant social and economic hardship. Although trypanosomiasis has been recognised for centuries, many aspects regarding how the parasite interacts with its host remain unknown. The primary goals of this research are: 1) to investigate how and why these parasites cause disease; 2) to determine what effect having other infections has on the severity of the disease; and 3) to discover new drug targets to combat the disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Human African trypanosomiasis is not only a devastating neglected tropical disease but can also be used as a tool to understand basic aspects of how our bodies combats infection. In our previous animal license, we generated significant knowledge regarding the mechanisms of skin colonisation, which is critical for parasite transmission. Additionally, we have generated a wealth of knowledge regarding the mechanisms underlying the damage to the brain caused by the parasites, including potential drug interventions. Moving forward, our work will shed light on how the parasite causes weight loss and sleep disturbances. This knowledge is critical to understanding this complex disease and has the potential to open up new possibilities for the diagnosis and treatment of infectious diseases. More broadly it can also provide insights into how our bodies fight infections, and the unintended consequences these processes might have on normal health.

What outputs do you think you will see at the end of this project?

Reaching the goals of this project will not only increase the current knowledge available regarding trypanosome infections, in the longer term our findings could lead to improved chemotherapy or disease interventions. This would greatly benefit, both socially and economically, the developing countries where this disease is endemic. Beyond trypanosomiasis the results of this project could shed light on other infection or conditions that affect the host during systemic chronic infections, including systemic metabolism and brain function.

Who or what will benefit from these outputs, and how?

In the short term, our results will have an immediate impact in the scientific community, including those investigating parasitic infections, as well as those investigating weight loss, brain function, and behaviour in response to infection. In the longer term, we also anticipate that our work will have an impact on disease modelling and potentially improved intervention strategies, including developing novel therapies.

How will you look to maximise the outputs of this work?

Our work is collaborative and interdisciplinary, and so we anticipate that the outputs of this work will be maximised further through ongoing and future collaborations. We will engage with the scientific community in parasitology, immunology, and neurology to disseminate the knowledge generated from our work. Additionally, we will publish the work obtained from all the animal work conducted under this license (including negative data) to reduce the use of animals in duplicated work elsewhere.

Species and numbers of animals expected to be used

- Mice: During the 5-year licence period requested it is estimated that a maximum of 10,000 mice will used.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice as they respond to infection in similar ways to humans and domestic animals such as cattle. Mice also have a well described immune system, genome and there is an array of mutant strains available, which will accelerate our research. The majority of trypanosome research studies have been based on this model and our results will be directly comparable to previous studies, maximising its impact.

We will use adult mice (>8 weeks old) as their immune system will be fully formed and functional.

Typically, what will be done to an animal used in your project

Typically, this project involves injecting mice with trypanosome parasites and monitoring how the infection progresses in the animal by routinely taking blood samples from the tail vein. Typically, wild- type mice will be infected for 1-3 weeks and may receive immunomodulatory substances via injection that may ameliorate the disease or shed light on the disease process. Typically, mice will be closely monitored daily throughout the procedures and supportive treatments such as soft food will be provided, if necessary. Typically, colonies of genetically altered mice will be bred and maintained and then adult mice infected for 1-3 weeks via injection. In a minority of cases, surgery may be performed to implant a wireless subcutaneous or intradermal telemetry device. In some instances, we will irradiate mice or treat with chemical compounds to modify the bone marrow, altering the immune system.

What are the expected impacts and/or adverse effects for the animals during your project?

In the majority of cases infection by trypanosomes may induce mild adverse effects such as the transient pain associate with injection or periodic mild clinical effects of the infection, such as the development of a rough / stary coat or lethargy. In some cases, the mice could develop more moderate clinical signs including staggering when walking. If no signs of clinical improvement are noted during this period, the animals will be humanely killed. It is also possible that a few animals may decline considerably becoming lifeless, if this does occur the animals will be humanely killed. In rare instances, immunosuppresion may occur as a result from irradiation. This could result in unintended side effects such as

opportunistic infections. In such rear events, animals will be humanely killed to avoid unnecessary suffering.

Extensive experience of this infection model and a familiarity with the techniques used, allows us to recognise quickly any unexpected adverse signs. All mice will be closely monitored (at least every three days) throughout the procedures and recorded on the animal sheets and endpoints implemented as required. All animals will be humanely killed on completion of the experimental procedure.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

mice: mild 20%

mice: moderate 80%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Trypanosomes can be maintained in culture flasks and this technique will be used to create genetically modified parasite lines to study genes that may influence parasite survival and in initial drug investigations. However, trypanosome infection cause changes in multiple interconnected systems and organs within the host and neither the development of the disease nor the ultimate success or failure of potentially useful treatment strategies can be investigated fully in the isolation of a culture flask. For example, when the effects of trypanosomes were examined using a culture model of the blood-brain barrier no lasting harm to the integrity of the barrier was detected. When this was investigated using a mouse model of trypanosome infection, a progressive increase in barrier impairment was associated with disease development. This clearly illustrates a disparity between the mechanisms at play in tissue culture models and animal models of this disease.

Which non-animal alternatives did you consider for use in this project?

Human tissue obtained from stem cells generated on a dish (known as organoids), skin explants, and endothelium monolayers.

Why were they not suitable?

Although these models are promising, as they stand, they do not fully recapitulate the responses obtained in animals. For example, brain organoids are devoid of innate immune cells, which limits its utility in the context of modelling infections in living animals. We are and will continue to invest efforts to keep developing organoids into more suitable culture models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Analyses of the data gained from the experiment will usually involve a factorial design to ensure that the greatest amount of information is achieved from a small number of animals. In general, the number of mice required to achieve the goals set out in this project licence has been calculated using an experimental group size of 6. Additional statistical support will be available when required to ensure that we gain the most accurate data using the minimal number of animals. Breeding colonies will be maintained at a minimum size.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

If appropriate, factorial designs will be followed to ensure that the greatest amount of information using the lowest number of animals is achieved. In addition, where possible, experimental groups of animals will be used to fulfil two objectives, e.g. pathology or gene expression studies can be performed using material harvested from animals assigned to imaging studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When appropriate, we will conduct pilot experiments to determine appropriate sample size and power calculations. We are also building a tissue bank repository in the lab, obtained from previous experiments, that can be used as an alternative to conducting repeated experiments. Our breeding system ensures that littermates are used either in experiments or as breeders. As a general rule, we will maintain our colonies in low numbers and will

only expand it when needed, and only for a finite period of time. At all times, we will follow the NC3Rs PREPARE guidelines and Norecopa guidelines.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the most suitable laboratory animal to use in this system as trypanosome infections can be manipulated to reproduce each of the disease phases that are essentially similar to those found in both human and animal trypanosomiasis. In addition, the mouse provides a standardised animal with a wide range of analysis reagents and genetically altered research lines available. Where possible, we will use chemical compounds such as Busulfan as alternative, less aggresive, methods to irradiation in order to manipulate the immune system.

Extensive experience in the use of this mouse model provides familiarity of handling and maintaining infections and in the consistent induction of the various stages of the disease. This also results in a reduction in the severity of the procedures performed and in the number of animals required to achieve the goals of the experiment. To minimise the impact to welfare all animals will be closely monitored throughout the procedures and, if necessary, for those animals that require it, supportive treatments, such as soft food will be placed within easy reach.

Why can’t you use animals that are less sentient?

Trypanosomes are able to infect many vertebrate hosts, including mammals and fish. Whilst it is possible to infect zebrafish with trypanosomes, this requires the development of intricate infrastructure and husbandry of large fish such as carp to maintain the life cycle of the parasite. Further to this, it remains unclear whether zebrafish can be used to model the disease pathologies experienced by infected humans. Due to the extended nature of rodent models of infection, it is not possible to limit infection to an immature life stage. Moreover, most humans are infected during late adolescence or adulthood, when they have a mature immune system, meaning that juvenile rodents would not accurately reflect human disease pathology.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have and will continue to adopt NC3Rs and local guidelines to handling methods, housing conditions, and environmental enrichment, to ensure the welfare of the animals in our license. Our procedural approaches have been refined over the years to ensure that volume size, frequency of inoculation, and routes of administration (including needle size) do not cause distress to our animals.

We typically monitor the animals after procedures to ensure there are not unwanted procedural side effects. We do not require analgesia but will maintain an open dialogue with the local veterinary team to make sure this is incorporate in a timely manner if needed.

Animal monitoring and pain management are an important part of all the procedures within the project. The need to minimise suffering is always considered when planning experiments and we routinely revise our experiments to reduce animal suffering. When using irradiation which can cause side effects in the gut, we will develop a more targeted approach thus improving the welfare of the animals, for example, by using alternative chemical reagents with less unwanted side effects (e.g., Busulfan). Of note, none of our experiments exceed a moderate severity level.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will ensure our experiments are designed in accordance with the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will engage with local authorities to ensure the work conducted under this license aligns to ethical standards, maintaining the 3Rs principles at the core of our activities. To this end, we will participate national meetings organised by the establishment and National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) in addtion to in-house 3Rs meetings and events, to incorporate changes to our procedures in an effective and timely manner.

72. Models of Mitochondrial Diseases: Pathogenesis and Therapy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Mitochondrial disease, Model generation, Gene therapy, Experimental therapy

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To generate and maintain mouse models to understand mitochondrial diseases and and develop effective treatments.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Mitochondrial diseases arise from harmful changes (i.e. mutations) in mitochondrial and/or nuclear DNA, which produce molecules important to normal cell function. These mutations cause changes in the structure and function of mitochondria, which disrupt the normal mitochondrial processes such as energy production (via oxidative phosphorylation), mitochondrial replication and removal (via fission and fusion), and ion transport across the mitochondrial membrane (important for normal cellular functioning). Impairments in one or more of these mitochondrial processes present in patients as diverse metabolic disorders which are difficult to treat, due to the wide range of possible signs and symptoms of these diseases. The work proposed within this licence aims to understand the mechanisms underlying mitochondrial diseases and develop more targeted treatments (such as pharmacological and non-pharmacological therapies).

What outputs do you think you will see at the end of this project?

From the work undertaken in this project, we expect three main outputs:

1. New mouse models of mitochondrial disease which carry mutations similar to those found in humans. At present, there are only a handful of mouse models which replicate the signs and symptoms experienced in patients with mitochondrial disease. More models are required to support outputs of points 2 and 3.

2. The molecular, cellular, and behavioural characterisation of the resulting models, that will grow our understanding of mitochondrial function and inheritance both in health and disease.

3. Potential treatments for correcting the dysfunctional mitochondrial in mice and provide initial data for future clinical translation. At present, there are no cures for mitochondrial diseases.

Outputs from this work will support future applications for funding and potential patents. Outputs will be shared with other researchers in the field via publications in scientific journals and presentations at scientific meetings.

Who or what will benefit from these outputs, and how?

In the short and medium term, the models generated will be used to expand our knowledge on the basic mechanisms of mitochondrial biochemical pathways and the impact of altered gene states in the execution of these pathways. The models developed will be characterised and samples will be collected and made available to internal research groups and external collaborators. The knowledge gained will be used to develop and test of novel therapeutic interventions. This is done with the long- term view of opening new avenues of therapy for patients with mitochondrial diseases.

How will you look to maximise the outputs of this work?

This output of this work will be shared with both national and international collaborators, as well as published in relevant scientific publications.

Species and numbers of animals expected to be used

- Mice: 22675

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice have the closest genetic relationship of a model organism to humans. They have a short lifespan which allows researchers to study animals throughout development and adulthood within a relatively short time. In addition, mice are prolific breeders. Over recent decades, the genetic material of mice have proven to be manipulatable using gene-editing technologies.

Mitochondrial diseases can occur at any age. Therefore, we propose using animals across the life stages, from embryo to adult, to study dysfunctional mitochondria, depending on the hypothesis being tested (e.g. inheritance of mutated mitochondrial DNA or accumulation of mutations during adulthood).

Typically, what will be done to an animal used in your project?

Substance administration: Animals used in this project may undergo injection of chemical and/or biological substances, with or without anaesthesia, surgical procedures, sampling of blood/cells/tissues on multiple occasions, and/or administering drugs via food or drinking water. Substances may be given by injection to the eye, vein, muscle, or directly into the brain.

Behavioural testing: Animals used in this project may undergo behavioural testing [e.g. testing muscle function using a treadmill test and/or testing the function of the eye using electro-oculography (EOG), etc.] to characterise the functional differences resulting from the mutation in the mouse mitochondria.

Animals may undergo a combination of both substance administration combined with behavioural testing.

The majority of animals will undergo five or less regulated procedures during the course of an experiment.

It is estimated that the majority (approx. 80%) of animals undergoing regulated procedures will be juvinelle (up to 8 weeks old) and adults (more than 8 weeks old). The remaining 20% will be neonates (new born pups up to weaning - approx. 3 weeks old).

Experiments will vary in duration from days to months. At the end of breeding or experiments, animals will be humanely killed and tissues collected for further study.

What are the expected impacts and/or adverse effects for the animals during your project?

Due to the disruptions in metabolism caused by the gene mutations and/or the therapies applied, some animals may experience symptoms such as weight loss, abnormal movement (e.g. uncoordinated gait), altered behaviour, and possible pain and discomfort. In most cases, pain, stress and discomfort will be kept to the minimum and, if present, it will be mild and transient. Where stated in the licence, pain will be alleviated by giving analgesia. Animals exhibiting behavioural and motor differences may suffer Moderate severity due to the impact of these symptoms on their normal behaviours in the cage, including feeding. Animals experiencing Moderate severity will be closely monitored for weight loss (to gauge the impact of the abnormal movement on their ability to feed) and will be killed immediately if >20% of their baseline body weight is lost. In most cases, weight loss to this degree is observed over weeks. Animals that display ataxia (i.e. observed as a wobbly, lurching gait) for more than 24hrs will be killed immediately.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: Sub-threshold 33.3%

Mild 57.27%

Moderate 9.43%

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal experiments remain a necessary part of our research as mitochondrial diseases affect multiple tissues in the body (e.g. brain, muscle, liver). In order to develop mouse models that mimic human patients with mitochondrial disease, the whole animal is

required to monitor for signs of disease. This multi-system affect cannot be replicated in vitro (i.e. in isolated cells in the lab).

In addition, the gene therapies we develop need to be tested for target specificity when delivered systemically (i.e. administered into the blood via injection of a vein). This currently cannot be simulated in vitro and is a vital step towards translation of our novel therapies to treat patients.

Which non-animal alternatives did you consider for use in this project?

Mouse adult fibroblasts (MAFs) and mouse embryonic fibroblasts (MEFs) may be harvested and used to generate cells which can be studied in vitro, reducing the number of animals used in some cases. Prior to testing in animals, gene editing therapies are tested in vitro, using established mouse cell lines, for their efficacy prior to testing in vivo (i.e. in a living animal, in this licence the animals used are mice).

Why were they not suitable?

The study of cells in vitro does not simulate the environment of the entire body. Hence, in vitro cell culture can help to refine treatments prior to in vivo testing but cannot replace the interaction of all the tissues at once, as in the whole animal. Cells cannot be used to study animal behaviours which may be perturbed in mitochondrial diseases, for example walking, feeding, or vision.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For mitochondrial disease models, the estimated animal numbers are based on our past experience, and reflect the challenges of maintaining a colony of female mice transmitting mitochondrial DNA (mtDNA) within the desired ranges. The effect of unknown mtDNA selection during gestation contributes to the instability of mutation levels in a mouse litter. Maintaining mutation levels within the desired experimental range requires a larger number of breeding pairs due to changes in mutation level during transmission.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The PREPARE guidelines are considered prior to experiments, as well the NC3R's Experimental Design Assistant. These guidelines include calculation of required sample

size for the chosen analysis method and careful planning of colony expansion to ensure any surplus of animals generated is kept to a minimum. Where possible (e.g. animals of appropriate age and sex are available) we will source wild type animals from the 3Rs enquires list for this project.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Colonies will be reviewed routinely and their size adjusted according to experimental need. Wild type mice will be bought in when needed, removing the requirement for keeping a wild type colony.

Pilot studies will be conducted for planning complex dosing, that require initial in vivo validation and optimization.

In the majority of experiments, it is not optimal to randomise the animals selected for treatment vs control groups. In most cases, we want to study the animals with the highest levels of mutant DNA. Blinding is possible at the point of administration (e.g. substance administration), however blinding may not be effective due to the resulting changes in the animals health or behaviour during the course of the experiment.

We will use a superovulation protocol to increase embryo yields. This results in a reduction in the number of females used, as we can collect more embryos for study. In addition, this will allow the colony to be kept smaller as we will not require as many females with the desired genetic profile.

Whenever possible (e.g. animals of appropriate age and sex are available), we will endeavour to source wild type animals from the 3Rs enquires list.

Whenever possible, we will co-ordinate internally to make spare animals/tissues available to researchers within our department. In addition, we are listed on a 3R's enquiry list, where we shall list surplus tissue/animals for other researchers to use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used to model human mitochondrial diseases. The majority of these mice display mild symptoms of diseases. Mice are the least sentient animals available that share very similar anatomy and mitochondrial functions as humans.

Why can’t you use animals that are less sentient?

The life stages used under each protocol have been carefully considered and meet the experimental life stage need (e.g. early interventions for mitochondrial diseases need to be given to neonatal pups, as therapies may be more effective early in life).

Mice are good breeders, producing offspring with the desired genetic profiles in a feasible time span to allow for experiments to be conducted within the time frame of the licence. Live animals are required to test in vivo therapies. Wherever possible, animals will be killed prior to tissue harvest.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals undergoing substance administration will be monitored daily, in some cases twice daily (AM and PM). Following a regulated procedure, a post-procedure welfare check will be scored using a procedure-specific check sheet, to assess the body condition, feeding behaviour, and (e.g.) site of administration. The procedure-specific score sheets will be developed with the advice of the NACWO and/or NVS. Adherence to the welfare check sheet will ensure that the humane endpoints are adhered to and the assigned severity levels are not exceeded.

Where required, peri-surgical analgesia (i.e. pain relief) will be administered to animals, with the use of voluntary treatments such as medicated flavoured jelly, paste, or milk shake liquid.

All users are trained and encouraged to use methods of handling of the animals to reduce stress (e.g. hand cupping or use of tunnel).

Wherever possible (i.e. in the majority of cases), animals will be group housed. In the minority of cases, it may be unavoidable that males are singly-housed. For example, stud males cannot be group housed due to fighting, which may cause greater suffering than single-housing. In addition to the group housing of animals, enrichment of the home cage will include suitable size cages for the animals age/size, including contents such as tunnels and nesting material, as well as a mezzanine level and freely available food and water.

Where the genetic mutation affects the ability of the animal to feed, for example due to altered behaviour/motor impairments, feeding may be assisted by providing water gel, food, and/or mash diet on the cage floor.

All wild type animals brought into the animal facility will be held for 15 days acclimatization and health monitoring before undergoing a regulated procedure.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The following guidelines are referred to and considered prior to starting new projects:

- The PREPARE guidelines, which consists of planning guidelines for in vivo experiments, will be considered prior to starting experiments.
- LASA (Laboratory Animal Science Association) guiding principles, which provide up-to-date guidance on training and education for PIL holders, aseptic surgical practices, good record keeping.
- RSPCA guidance on Research Standards and the role of the 3R's in improving the planning and reproducibility of animal experiments.
- National Centre for the 3Rs (NC3Rs) resources on experimental design, genetic modification, handling, husbandry, welfare assessment, and in vivo techniques.
- Breeding resources from sources such as Jackson Laboratory, The International Mouse Phenotyping Consortium (IMPC), International Society of Transgenic Technologies, and the International Mouse Strain Resource (IMSR).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The PPL holder and PIL holders associated with the licence are subscribed to the 3R's enquiry email list. The Named Information Officer sends regular emails with updates on regulatory guidelines and available resources. In addition, the PPL holder routinely attends events organised by the NC3R to keep up to date with the latest developments in animal welfare, experimental design, and new guidelines. The PPL holder attends national and international conferences on animal welfare and the use of animals in scientific research (e.g. Federation of European Laboratory Animal Science Associations 2022 conference in Marseille, France).

73. Pk and Pkpd Studies to Support Respiratory and Immunology Research

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Pharmacokinetics, PKPD, Respiratory disease, Immunological disease

Animal types	Life stages
Mice	adult, aged
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This licence has two aims:

To enable us to decide what doses of new treatments we need to use in our respiratory and immunology disease models. We will do this by dosing animals and taking blood samples to measure how much of a new treatment is present at certain times after dosing (pharmacokinetics (PK))

To enable us to understand how much of a treatment is needed to have an effect on the biological target. We will do this by dosing animals which have had a simple pretreatment or challenge, so that we can measure both the amount of treatment in the blood (PK) and

the effect of the treatment (pharmacodynamics (PD)) and use both pieces of information to build a mathematical model (PKPD model). This can be used to inform both future animal disease studies and human dose selection for clinical studies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There is an ongoing need to discover and develop new treatments for respiratory and immunological diseases such as asthma, chronic obstructive pulmonary disease (COPD), arthritis and lupus. To bring these life changing medicines to patients takes many years of research and requires demonstration that these treatments are effective and safe. To understand how effective a new medicine could be in human disease we bring together a package of information that shows how it affects human and animal cells, how it interacts with tissues from patients, how it modifies relevant animal models of disease and how much we will need to give to patients to be effective. Before testing a new treatment in an animal model we need to know how much to give and how often to have an effect. This licence will enable us to test new treatments in mice and rats and predict how much to use in these models.

This information also allows us evaluate the doses that we need to deliver to be effective in patients.

What outputs do you think you will see at the end of this project?

This Licence will provide key information about the properties of our novel treatments, such as how long they remain active in the blood of an animal after dosing, which is needed by our research teams to plan and deliver further studies. For example we will obtain an understanding of the peak concentration in the blood, which parts of the body it reaches and how long it takes to be eliminated. These findings along with information from human and animal cell studies will enable us to move suitable novel treatments forward through our pipeline and into human clinical trials, and ultimately result in new medicines to treat patients. Pharmacokinetic data are often reported when we publish results in disease models.

Who or what will benefit from these outputs, and how?

As an organisation our focus is on delivering life changing medicines to patients, and this licence will contribute to that overall goal by generating specific information that is used to determine how much medicine needs to be dosed to have an effect. Without an understanding of how much to give or how often, we cannot conduct effective research in animal models of disease or predict how much we may need to give patients. Our studies

are used to support specific research into new treatments for diseases that affect many patients and have life limiting impacts, including chronic lung disease and immune disorders.

Drug discovery and development can take years if not decades, so novel treatments tested under this licence are likely to enter human clinical trials after the 5-year lifetime of the licence. However, treatments tested under previous licences with the same goal are now in clinical phase testing and we are confident that this will continue.

How will you look to maximise the outputs of this work?

We have an open culture and interactions between our scientists and those from other research organisations are encouraged. Whenever possible we regularly share our findings at academic conferences and symposia, but due to the commercially sensitive nature of the work we are unable to be open about our very early and novel findings until appropriate patent protection is obtained. We do share openly information regarding our animal study techniques and practices with our peers to maximise animal welfare, and are committed to supporting the 3Rs.

Species and numbers of animals expected to be used

- Mice: 4600
- Rats: 2600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The overwhelming majority of these studies will be performed in adult mice, as this is the species most commonly used in the disease model studies we will carry out. Mice are used in those models as they are a mammalian species with a similar (but not identical) organ anatomy and immune system as compared to humans. Although immunological and respiratory disease can affect patients at any age, the majority of rodent studies are performed in adult individuals (ie post-pubertal) as rodent development is very rapid and animals become sexually mature in a matter of weeks. Rats will be used rarely, when the disease model is performed in the rat, or there is some other technical need (such as the target system in the rat being closer to the human in its properties).

Although it will be very rare, we may occasionally need to understand the pharmacokinetics of our new treatments in older animals, which for mice and rats equates to 1 year or older. This will only be done where there is a disease model that requires it.

Typically, what will be done to an animal used in your project?

In a typical pharmacokinetic experiment, adult mice would be implanted with identification microchips under anaesthetic and a few days later weighed and dosed (the dose given being related to the weight of the animals). The dose may be an oral liquid, delivered using a round ended dosing tube attached to a syringe which is placed into the throat of the animal to ensure all the material reaches the stomach. Alternatively, the dose may be given as an injection using a hypodermic needle under the skin, or into a vein or into the abdominal cavity. Animals are returned to their cages and then at set times (which could be over a single day or a number of weeks) taken out and restrained in a small tube for a few minutes while a blood sample is taken from the tail using a small hypodermic needle. Usually no more than 3 samples are taken from the tail vein in the lifetime of the animal, and only if the samples needed are very small. The final blood sample is usually taken under deep anaesthesia, when a hypodermic needle is placed directly into the heart to take a large volume of blood, followed immediately by killing the animal. Apart from these typical experiments we may occasionally dose using other techniques such as directly into the lungs, or injection into a leg muscle, or surgical implantation of a drug delivery pump under the skin.

Some studies may also include giving the animal stimulus (such as an injection of a substance that can trigger release of inflammation markers into the blood) so we can understand activity of the treatment as well as exposure (PKPD studies). These may look similar to a pharmacokinetic study but with the additional injection of the stimulus or other pretreatment.

What are the expected impacts and/or adverse effects for the animals during your project?

Animal dosing may cause some brief pain, such as when a hypodermic needle is used to dose under the skin or into a vein or the abdominal cavity. This is brief in nature and animals are not expected to show any signs of ongoing discomfort afterwards. Similarly, the needle used to take a blood sample is likely to result in fleeting pain or discomfort but should not have a lasting effect. Direct administration of material into the lungs may be done under anaesthetic by introducing a narrow tube to the airway, and this has a small risk of touching the sides of the airway and causing irritation or injury.

The treatments we will dose are not expected to result in any adverse events, and for novel materials that have not been in animals before we will run a small pilot study (tolerability test) to check that this is the case. In studies where we will use a challenge or stimulus some animals may exhibit clinical signs associated with inflammation and illness such as reduced grooming, reduced social interaction and lowered body temperature.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority (>90%) of animals are expected to experience a severity of Mild. In our experience, simple PK and PKPD studies with dosing of a novel therapeutic, followed by tail vein sampling, do not result changes to animal condition or behaviour that are greater than Mild.

The remaining animals (<10%) may experience Moderate due to recovery surgery for implantation of drug delivery pumps under the skin, or in the event that animals on the tolerability testing protocol experience greater than Mild severity. Animals on the PKPD protocol may also experience Moderate changes such as reduced grooming or activity over a few days.

These proportions are estimates and may change in either direction if for example the projects we progress in the next 5 years require more or less drug delivery pump work.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is not yet technically possible to accurately predict the pharmacokinetic properties of novel molecules, so animal studies are required. Much effort is put into screening out unsuitable molecules during the early stages of research, for example using human and rodent liver cells to see if they are rapidly broken down, and other cell based assays to estimate how easily they might be absorbed from the stomach into the bloodstream. This greatly increases the likelihood of success, in terms of seeing good blood levels after dosing, in our animal studies. The complete characterisation of new molecules that is needed to calculate appropriate doses for use in animal models of disease, or to guide first time in human dosing schedules, still requires us to use animals.

Which non-animal alternatives did you consider for use in this project?

There are multiple stages of non-animal screening of new molecules that are in place before testing in rodent. Depending on the type of new therapeutic agents will have performed tests (both using computer modelling and lab testing) to understand the physical properties which can affect drug absorption and how rapidly it could be broken down in the body. Cell based systems are used to assess how for example liver cells might process and break down a molecule.

Why were they not suitable?

The information gained from these non-animal tests cannot account for the complex interactions that take place inside a whole animal with multiple organs, cells and enzymes which could affect the way a drug molecule moves around the body, is processed or broken down and is eliminated from the body. As these molecules are going to be tested in animal models of disease, it is a scientific and ethical imperative that we select doses (how much treatment, how often to dose, which route to dose) which are likely to give us the necessary information. These studies can also be used to guide selection of doses for human clinical trials when combined with other information such as the way human cells or enzymes interact with the molecule.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These estimates arrived at based on current and historical usage (how many animals per study, how many studies of each type per year) and allowing for anticipated changes in demand.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Pharmacokinetic studies are usually not hypothesis testing in a statistical sense, so we do not test for a significant difference in blood levels per se. Instead we are interested in obtaining parameters such as the peak concentration in the blood, and how much time it takes for the blood levels to drop (so we can understand how much drug to use and how often to dose). The number of animals per study is therefore a function of how many samples are required, and for how long, and the number of replicate samples required at each time-point, which is normally n=3.

We normally take more than one sample per animal (the limiting factor is blood volume, we do not want to impact animal welfare by sampling too much), so our analysis benefits from replicate samples taken from the same animals. Standard protocols normally require only 3 samples per time point, although this may be increased in certain situations for example when PKPD studies are performed and in these situations statistical methods are used. For some types of new therapeutics we may be able to dose a mixture of different molecules and measure them all simultaneously, which further reduces the number of

animals. This cannot be done as a standard method as there is a risk of interactions or technical limitations on the measurement technique.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The animals we use for these studies are usually standard strains obtained from commercial suppliers which maximises the efficiency of production and animal usage. Occasionally we will use genetically altered animals bred for scientific use and the numbers are carefully monitored to ensure overproduction and wastage is minimised. New types of therapeutic agent which have not been tested in animals before are tested under a tolerability protocol on this licence, which uses very low number of animals (usually n=2) to confirm that these molecules are suitable for use in animals and do not induce any unexpected effects. This reduces the likelihood of us proceeding to a full study only to find a molecule causes an adverse effect that would stop the experiment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Pharmacokinetic studies normally consist of dosing animals (using a hypodermic needle and syringe, or special tube for dosing into the stomach), followed by taking blood samples, normally from a vein near the surface of the skin on the tail. Different dose routes may be required for different types of new molecule, or to determine if the molecule can be absorbed from the stomach, which may be important for some projects. Sometimes we may need to deliver a constant amount over a period of time, and in these cases we may use specialised miniature drug delivery pumps which we will position under the skin surgically. The use of these pumps may be needed to maintain a steady blood level or to avoid having to give multiple injections.

Why can’t you use animals that are less sentient?

As these studies are used to help design experiments in animal models of disease we need to have high confidence that the doses we pick are relevant. Using species that are less sentient introduces a significant risk that the information we obtain is not relevant and could lead to wasted animals in the disease models. Terminal only studies would be of limited duration and risk giving information that is compromised as for example blood flow

to the liver and other organs may be different under anaesthesia and could affect the measurements, and the presence of anaesthetic could alter the way material is processed by the body.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals are kept in modern well equipped facilities staffed by experienced and motivated scientific and welfare personnel. Animals are checked at least once daily when not on a study and at least twice daily once dosing and sampling have started. When surgery (under general anaesthetic) is used for implantation of drug delivery devices, peri-operative pain relief (analgesia) will be given as standard.

Hypodermic needles are always discarded after a single injection so that blunted needles (which can cause unnecessary tissue injury and pain) are not used.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

LASA Good practice guidelines; AAALAC programme

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As a Project Licence holder I am engaged with local and national 3Rs groups and events and am kept informed by my NIO of relevant new information.

74. Control of Brain Circulation and Metabolism in Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

brain, metabolism, cerebral blood flow, dementia, neurodegenerative disease

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant
Rats	adult, juvenile, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The high metabolic rate of the brain, associated with the activities of billions of nerve cells processing information, requires constant and optimal nutrient and oxygen supply, as well as effective elimination of metabolic waste products. There is growing evidence that reduction in the blood flow to the brain, resulting in impaired delivery of metabolic substrates, damages nerve cells and the connections between them, leading to cognitive decline, and the development of neurodegenerative disease, such as Alzheimer’s disease. We will examine fundamental biological processes that ensure nutritional support of nerve cell activity, effective elimination of CO2 as a major brain waste product, and the mechanisms activated in conditions of metabolic emergency, such as when oxygen supply to the brain is reduced.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This research is expected to contribute to our understanding of how computational machinery of the brain is fuelled and protected from deleterious accumulation of carbon dioxide and/or reductions in oxygen supply. This new knowledge may prove to be important for future development of novel preventive and therapeutic strategies designed to maintain cognitive health and promote brain longevity.

What outputs do you think you will see at the end of this project?

This research is expected to make a significant contribution to our understanding of the brain. The experiments are designed to investigate the fundamental mechanisms responsible for metabolic support of neuronal computation and the primary output of this research will be new scientific knowledge. All results will be published in scientific journals and immediately available to the scientific community and general public.

Who or what will benefit from these outputs, and how?

There is growing evidence that a reduction in brain blood flow, impaired cerebrovascular reactivity, progressive acidification, and metabolic insufficiency precipitate neuronal damage, contribute to cognitive impairment, and the development of dementia and neurodegenerative disease.

Understanding the mechanisms of neuronal activity-dependent control of brain blood flow, metabolic substrate delivery, and the innate adaptive mechanisms, recruited in response to acute metabolic compromise, may prove to be important for the development of preventive and therapeutic strategies to maintain cognitive health and promote brain longevity. The earliest beneficiary will be the scientific community, as our studies will inform future research. In a longer term, this research is expected to have an impact on drug development projects by the pharmaceutical industry and on the way how clinicians think about the diseases and treatment strategies.

How will you look to maximise the outputs of this work?

We always aim to maximise both the quality and impact of our research. We use the most appropriate and refined experimental strategies that provide clear, unequivocal results. This is achieved by keeping informed about the latest developments in our field of research and collaborating with the leading research laboratories around the world. We maximise the impact of our research by communicating the data to the scientific community via conference presentations, invited seminars and publications in the leading general science and specialised peer-review scientific journals. Large datasets will be uploaded to public data repositories and/or available to other researchers upon request.

Species and numbers of animals expected to be used

- Mice: 2500
- Rats: 1200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The fundamental questions addressed by this project are common to all mammalian species, including humans, therefore a mammalian species is required for the study. Rodents are usually chosen for the research of this type as the lowest species which accurately mimic human physiology and pathology, in order for the research data to be relevant to human disease. Therefore, laboratory rat and mouse have been used extensively to study the brain function, brain energy metabolism, and the mechanisms of cerebral blood flow control in health and disease. Most of the experimental data addressing these mechanisms were obtained in studies using rats and mice. In addition, the use of mice allows the use of transgenic technology for cell-specific targeting and blockade of the physiological signalling mechanism(s), which is not usually possible to reliably achieve by other experimental approaches currently available. Thus, the laboratory rat and mouse are the most appropriate models for the proposed programme of research, designed to investigate the mechanisms that maintain the brain's metabolic homeostasis.

Typically, what will be done to an animal used in your project?

The majority of our experiments are performed under deep terminal anaesthesia. This experimental model is the least severe and is proven by our track record to produce high quality, physiologically meaningful and significant results. Some protocols of this project involve surgical procedures involving genetic targeting of brain cells to express proteins designed to modulate or monitor the cellular function and/or implantation of the recording devices for non-invasive physiological monitoring. These surgical procedures typically last ~30 min and are not unduly traumatic for the animals; after a rapid recovery from anaesthesia/surgery they eat, drink and gain weight in a similar manner to non-operated littermates. Pre- and post-operative analgesia and antibiotics are given. Not more than two surgical procedures with recovery will be applied. These will be followed by either non-recovery procedures or procedures which do not result in pain, suffering, discomfort or lasting harm. At the end of the experimental studies the animals are humanely euthanised.

What are the expected impacts and/or adverse effects for the animals during your project?

The transgenic mouse strains/lines that we use do not show any adverse/harmful phenotype. A small number of the experiments will involve treatment of mice with

tamoxifen to modulate gene expression. There is some evidence that intraperitoneal administration of tamoxifen can potentially cause weight loss, particularly in younger mice. To avoid these potential adverse effects, older animals (3-4 months old) will be used. Wet diet and/or high energy, palatable food will be provided during the course of treatment to mitigate any potential weight loss.

The adverse consequences of the viral manipulation of the neural circuitry under investigation are expected to be benign. Work under the previous licences has shown that the main adverse effects are short-lived, and result from the surgery itself. The animal may suffer from post-operative pain (alleviated by painkillers) for up to 24h after guided microinjections into the cerebral cortex, and for up to 72h after the microinjections into the brainstem (because this involves dissection to the neck muscles). Similar recovery times are seen after the implantation of the cranial windows, or implantation of telemetry devices to monitor blood pressure and heart rate and electrophysiological recording devices. After this time no differences in behaviour are observed when post-surgical animals are compared to surgery-naive littermates.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority (>90%) of animals used under this project are experiencing 'mild' severity or undergo "non-recovery" experimental procedures. Breeding of transgenic strains is per definition 'mild'. The only invasive procedure is ear notching for genotyping, and otherwise just handling of the mice for husbandry. The severity for those animals that are simply tissue donors or undergo procedures under terminal anaesthesia is "non-recovery", as they only experience induction of anaesthesia, and for those animals undergoing recovery surgery it is 'moderate', because of surgical trauma and the healing process. None of the other tests and experimental procedures the animals are undergoing prior and or after surgery produce any pain, discomfort, suffering or lasting harm that would require classification as 'moderate'.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Brain metabolic homeostasis is ensured by complex, multi-layered mechanisms controlling cerebral blood flow, metabolic substrate processing and delivery, maintenance of acid/base balance, and systemic mechanisms that regulate the circulation and breathing. The use of animals is essential in a programme of research of this type as the physiological mechanisms controlling brain circulation and metabolism are extremely complex and cannot be adequately described or studied in reduced systems such as tissue/cell culture or brain slices. In the experiments designed to study these fundametal mechanisms of the body we need to monitor brain activity as well as the key physiological variables (e.g. heart rate, arterial blood pressure, brain blood flow). In our studies we extensively use the reduced preparations such as tissue/cell culture or brain slices. These experimental models are very useful to study the biological interactions at the cellular and molecular level, but the physiological/functional significance of the identified mechanisms can only be determined by using the in vivo animal models.

Which non-animal alternatives did you consider for use in this project?

In this project all the hypothesised signalling mechanisms will first be explored by pharmacological surveys using reduced preparations (cell culture, acute brain slices). These experimental models completely replace the use of live animals. Initial selection of targets is guided by the analysis of gene sequencing data, already obtained by our laboratory, and also available from open sources (e.g. http://mousebrain.org/).

Why were they not suitable?

Full understanding of brain energy metabolism can only be achieved in experimental studies using live animal models, where the blood is delivered by systemic circulation, the blood-brain barrier is intact, the integrity of the neurovascular unit is preserved and all brain cells are interacting and functioning in their normal physiological environment.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The indicated number of animals to be used in this project is based on the numbers of animals used previously in the research projects of a similar type. The number of animals to be used over the duration of five years can only be an estimate and is designed to give a maximal number that will not be exceeded. The majority of experiments built upon each other, and thus it is rather difficult to accurately calculate the exact number of animals needed. This is often different for each individual study, which is always powered based on

the magnitude of the effects observed previously with similar genetic models and experimental designs.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experimental animals are used in our laboratory in well-defined and carefully executed studies. Our main preparation is the terminally anaesthetised mouse or rat in which we carefully monitor and maintain a range of key physiological variables such as blood pressure, arterial blood gases and pH and body temperature. We monitor central respiratory drive and cardiovascular homeostasis and often provide positive pressure ventilation with supplemental oxygen. These measures are essential to reduce variability within a sample and, therefore, to minimize the number of animals required to test a specific hypothesis. In a typical experiment the sample sizes in all experimental groups will initially be six animals, but these numbers may be increased if the data suggest that this would increase the likelihood of obtaining significant differences. From many years of experience, we can detect a physiologically significant difference with 6 animals/biologically distinct samples per group, if the treatments cause differences between group means that are as large as 2.25 standard deviations (SD). If the expected difference is as small as 1.75 SD, we increase the sample size to 9. Although, increasing the sample size per group to 15 would enable to detect a difference between means of 1.25 SD, it is doubtful whether these differences have a biological significance. Therefore, it is estimated that between 6 and 9 animals will be required in an average experimental group (α=0.01; power=90%). Carefully designed and executed in vivo studies that provide robust experimental data and published in a timely fashion, ultimately reduce the number of animals used in research by preventing other investigators from exploring unproductive territories in experiments using both the reduced preparations and in vivo models.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Hypothesised physiological mechanisms are first explored by pharmacological surveys using reduced preparations (cell culture, acute brain slices), with initial selection of the most likely targets guided by the analysis of gene sequencing data, already obtained by our laboratory, and available from open sources. Both male and female animals are used, therefore, almost all mice produced through breeding are used in the experimental procedures. Individual animals are subjected to more than one test each and after the in vivo studies their tissue is used for further in vitro analysis, thus reducing the overall number of animals used in this research project.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The majority (>80%) of animals used in this project are experiencing 'mild' severity or undergo "non- recovery" experimental procedures. Breeding of transgenic strains is per definition 'mild'. The phenotype(s) of the animals used in this project is expected to be sub-threshold and genotyping will generally be undertaken using surplus material from ear notching for identification. The severity for those animals that are simply tissue donors or undergo procedures under terminal anaesthesia is "non- recovery", as they only experience induction of anaesthesia, and for those animals undergoing recovery surgery it is 'moderate', because of anaesthesia used, surgical trauma and the healing process. None of the other tests and experimental procedures the animals are undergoing prior and or after the surgery produce pain, suffering, discomfort, or lasting harm. All animals are handled with care (e.g. tunnel handling) and kept in social groups in cages with environmental enrichment (e.g. running wheels, tunnels, small wooden objects).

Why can’t you use animals that are less sentient?

The fundamental questions addressed by this project are common to all mammalian species, including humans, therefore a mammalian species is required for the study. Rodents are usually chosen for the research of this type as the lowest species which accurately mimic human physiology and pathology, in order for the research data to be relevant to human disease. Therefore, laboratory rat and mouse have been used extensively to study the brain function, brain energy metabolism, and the mechanisms of cerebral blood flow control in health and disease. Moreover, all critical molecular biology tools including targeted genetic modifications have been developed and established in mice and rats. In our laboratory all key experimental electrophysiological and imaging techniques that are relevant to the current proposal have been established, tested and verified in rats and mice, thus eliminating the need for the methodology-setting additional experimental animal studies. Animal suffering is minimised or avoided by implementing the experimental routines with analgesia +/- anaesthesia as appropriate for the procedure. A significant proportion of the planned experimental procedures (~70%) will be conducted in terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal suffering is minimised or avoided by implementing the appropriate experimental protocols with analgesia +/- anaesthesia. Wherever possible, surgical invasive procedures are replaced with non- recovery protocols or procedures which do not produce pain, suffering, discomfort or lasting harm (e.g. novel object recognition test for the assessment

of memory function). The majority of the experiments are performed under deep terminal anaesthesia and therefore the severity limit for these protocols is “non-recovery”. These experimental models are the least severe and were proven by our track record to produce high quality, physiologically meaningful and significant results. When conscious animals are used, the animal wellbeing is monitored on a continuous basis for the duration of the experiment and frequently during post-experiment or post-surgery recovery periods. We continually refine and optimise our protocols for surgical anaesthesia/analgesia and post-operative care of particular importance, and regularly review these in conjunction with the Named Animal Care and Welfare Officer (NACWO) and/or Named Veterinary Surgeon (NVS) to ensure any updates to the best practice are promptly implemented. We have recently improved our post-operative care protocol to provide a warmed home- cage recovery environment for mice for the first night after surgery, and post-operative analgesia in an edible formulation without the need to perform repeated injections.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our experimental design and procedural practice will be informed by best practice guidance published from a variety of sources. In particular, we will utilise the comprehensive list of recommended resources curated by the BSU Named Information Officers, including information from the Home Office, NC3Rs, Laboratory Animal Science Association (LASA), and Federation of European Laboratory Animal Science Associations (FELASA).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institutional Biological Services Units (BSU) are very proactive in disseminating the latest advances and best practice guidance regarding the 3Rs to all users of their facilities. In addition to this excellent source of information, we keep ourselves regularly appraised of general and field-specific advances via social media (e.g. by following The National Centre for the 3 Rs twitter account and blog). Opportunities to implement new 3Rs advances are taken as soon as feasible, via formal and/or ad hoc online and in-person BSU training courses, and refinement of our specific experimental approaches

75. Breeding and Maintenance of Genetically Altered Mice

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Transgenic, Mouse, Breeding, Neuroscience, Behaviour

Animal types	Life stages
Mice	adult, neonate, juvenile, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this work is to, breed and maintain mice with genetic alterations and supply researchers with strains to examine how sensory information is processed by specialised neural circuits in the brain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Genetically modified animals, particularly mice, have a widespread use in neuroscience research and have been shown to be of great value in explaining the function of genes and pathways in a wide variety of biological, physiological and pathological processes.

What outputs do you think you will see at the end of this project?

Mice bred under this licence will be used to understand the function and connectivity of neuronal circuits in the normal and genetically altered mouse brain. This work will enhance and advance knowledge on how the brain processes information from the outside world and converts it into behaviour. This information could lead to the design of new highly selective drugs for treating neurological diseases such as epilepsy, Parkinson's Disease, Alzheimer's Disease, depression, schizophrenia and autism.

Who or what will benefit from these outputs, and how?

The establishment currently has around 10 research groups using mouse models and many of these need newly generated mice to fulfil their scientific questions.

How will you look to maximise the outputs of this work?

- Reduction in animal numbers by effective liaison with end-user projects, to ensure the appropriate strains of the desired specification are bred (best model for the research areas), with minimal wastage and sharing of animal lines and/or tissues by several research programmes.
- Reduced transport time for the animals, as fewer animals have to be brought in.
- Strict barrier controls ensure animals remain pathogen free for introduction into barrier accommodation and maintenance at a high health status in optimum environmental conditions.
- High health status, with direct benefit on welfare and science. The standard of facilities available for the work is high, and reflects a high level of investment
- Under the previous Project Licence, there has been a proven record of efficient generation and breeding of GAA strains and this licence will continue and improve the provision of high quality animals to the researchers

Species and numbers of animals expected to be used

- Mice: 101,500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice have been shown to be of great value in elucidating how sensory information is processed by specialised neural circuits in the brain, and are sufficiently close to humans to reveal principles of information processing.

All life stages will be required for breeding and maintenance. Adult animals will be additionally be required for the following;

- Superovulation to generate ova or blastocysts Transfer embryos into recipients
- To produce sterile male mice for mating to obtain pseudo-pregnant female mice to be used for embryo transfer, if required.
- Embryos will be required for the creation or re-derivation of genetically altered embryos to generate founder stock.

Typically, what will be done to an animal used in your project?

Genetically altered animals will be bred and maintained under this licence in order to understand processes involved in neural circuits and behaviour.

The various procedural steps involved may include:

1. Injection of hormones to increase egg production in female mice.

2. Female mice may have embryos implanted.

3. Vasectomy or import of genetically sterile male mice to allow these to be used to induce phantom pregnancies in females so they will receive embryos generated in other females.

What are the expected impacts and/or adverse effects for the animals during your project?

Each new strain generated will have a well described and expected profile; however, animals will be monitored for unpredicted adverse effects and profiles will be monitored.

Surgical procedures will be performed under anaesthesia, using pain relief and following aseptic methods to minimize risk of post-surgical complications. Anaesthesia will be carefully and regularly monitored to ensure that an adequate depth is maintained throughout any surgical procedure, and animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Mice will be monitored throughout all procedures.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mild: 95% of animals will have an expected mild severity, this includes all animals only undergoing breeding and maintenance

- Moderate: 5% of animals will have an expected moderate severity, only applicable to those undergoing surgical procedures

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

The different animal models maintained and bred under this licence will integrate the complete range of molecular, cellular, physiological and behavioural interactions necessary to fully understand how genetic modifications result in normal or abnormal processes, focusing on neuroscience.

Which non-animal alternatives did you consider for use in this project?

Not applicable.

Why were they not suitable?

Animal models are required for breeding and maintenance.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on the annual Return of Procedures from previous breeding project licence, current and projected use has been considered.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Use of Home Office "Efficient Breeding of Genetically Altered Animals Assessment Framework"

Encouragement of all researchers to use the NC3Rs "Experimental Design Assistant" for animals bred under and requested from the breeding licence.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- Breeding programmes will be agreed in advance and regularly reviewed to optimally meet anticipated demand.
- Breeding programmes will be optimised wherever possible to produce only the required genotype.
- Freezing of eggs/embryos and sperm of lines created in-house will be carried out. Archiving of lines will avoid wastage from the need to maintain colonies by continuous breeding.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use mice to breed and maintain genetically altered mice.

Why can’t you use animals that are less sentient?

Adult mice are required for breeding and maintenance, however immature life stages will be used for the generation of founder lines when creating or re-deriving genetically altered embryos. Less sentient species are not suitable for this project, as they are required to be sufficiently close to humans to reveal principles of information processing in the brain.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The mice will be cared for by dedicated, experienced animal technologists who have the expertise and skills required to breed mice. Welfare problems that may occur at an early stage will be monitored carefully to determine appropriate end points in consultation with experienced animal husbandry technicians and veterinary surgeons.

Surgical procedures will be carried out aseptically and according to best practice, and peri and post- operative analgesia will provided.

When mating to produce pseudo-pregnant female mice for embryo transfer, the import and use of genetically sterile males may be considered as a substitute for in-house, surgically produced sterile males.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

- Home Office "Efficient Breeding of Genetically Altered Animals Assessment Framework"
- Jackson Laboratory "Breeding Strategies for Maintaining Colonies of Laboratory Mice"
- Preece, C., Alghadban, S., Bouchareb, A., Moralli, D., Biggs, D. and Davies, B., 2021. Replacement of surgical vasectomy through the use of wild-type sterile hybrids. Lab animal, 50(2), pp.49-52.
- Garrels, W., Wedekind, D., Wittur, I., Freischmidt, U., Korthaus, D., Rülicke, T. and Dorsch, M., 2018. Direct comparison of vasectomized males and genetically sterile Gapdhs knockout males for the induction of pseudopregnancy in mice. Laboratory animals, 52(4), pp.365-372.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The project licence holder is a member of several professional bodies within the industry, and as a result attends regular meetings and well as receiving communications regarding animal science and the 3Rs.

The establishment maintains an internal 3Rs group in addition to a working Animal Welfare and Ethics Review Body, both of which regularly review animal work and disseminate key information.

The establishment has nominated Colony Managers within the animal care facility and laboratory groups, that coordinate and review breeding practices at regular interviews.

The Named Information Officer (NIO) at the establishment publishes a quarterly newsletter, informed by other named persons, recent publications, and key information sources (i.e. the NC3Rs, LASA), which directly addresses the 3Rs and disseminates this information to stakeholders.

76. Zebrafish as an Animal Model for Nystagmus and Vision Disorders

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

nystagmus, therapy, optokinetic response, vision development, zebrafish

Animal types	Life stages
Zebra fish (Danio rerio)	adult, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand the mechanisms of normal and abnormal eye movements in zebrafish by investigating genes involved in the development of systems that control eye movements and vision development. We will also identify drugs that

influence the development of these systems, including a disease called infantile nystagmus (wobbly eyes).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Nystagmus is the involuntary to and fro movement of the eyes, and it affects approximately between 2- 3 individuals per 1000 people. In humans, this is broadly classified into infantile forms (onset within 6 months on life) and acquired forms. Infantile nystagmus is a debilitating lifelong condition associated with poor visual and social function. Mutations in genes that control development of the light sensing organ (retina) are responsible for a large number of cases of infantile nystagmus. In humans there is limited knowledge about how infantile nystagmus develops and currently there are no curative treatments. Thus, there is a clinical need to understand how infantile nystagmus develops and what treatments can be used for this disorder. Patients and charities representing patient groups for nystagmus have highlighted that understanding the mechanisms of disease and identifying treatments are important to them.

The genes identified in humans known to cause infantile nystagmus and/or abnormal retinal development are also present in mice and zebrafish. Previous work in mice and zebrafish have shown that when these genes are made non-functional (knocked out or switched off), they can exhibit abnormalities of eye movements and abnormal retinal development. This suggests that zebrafish is an excellent model to further study how these genes control eye movements and visual system development. Once an animal model is established for infantile nystagmus, it will not only shed light into how nystagmus develops but also it will allow us to test drugs that might be beneficial for use in humans with nystagmus. Therefore, this work has the potential to directly address the research priorities set out by the patients.

What outputs do you think you will see at the end of this project?

1. Resources in the form of zebrafish lines for nystagmus

2. Dose-response data for novel candidate drugs

3. Publications on: (a) techniques for eye movement characterization in zebrafish (b) mechanisms of infantile nystagmus and (c) candidate drugs for this disorder

Who or what will benefit from these outputs, and how?

1. Scientist interested in the mechanistic basis of nystagmus. We will share the mutant zebrafish lines with any interested party (by providing researchers with embryonic offspring

of the relevant adult fish). We anticipate this will be a valuable resource to facilitate further research into the pathogenesis and search for treatments for nystagmus.

2. We will publish and share the dose-response data from our drug studies. This will have significant value to other nystagmus research groups, scientists using zebrafish eye tracking and drug studies.

3. Patients and families who suffer from nystagmus. We will use our model to search for novel treatments for this disease, focusing on small molecules that already approved for use in human patients, to speed up the use in human patients.

4. The general public are fascinated to learn how the brain controls behaviour, including visual processing. We will present our findings at regular outreach events and via social media platforms.

How will you look to maximise the outputs of this work?

We will aim to publish our data in top tier scientific journals. In addition to scientific publications, we will disseminate our findings through social media (twitter) and conferences. Similarly, we have close links with patient advocacy groups and regularly hold patient/public involvement events. These additional platforms to share our findings will ensure maximal output for this work.

We will also deposit all our findings (including negative findings) in pre-print servers such as bioRxiv.org thus providing useful early open access data on both successful and unsuccessful experiments and approaches.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 8400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Zebrafish are the best choice of model organism for this study. They are easy to keep, freely available and breed prolifically. Zebrafish can also be used to model eye movement disorders. They have similar eye movement characteristics as humans and the neural systems that control eye movements develop rapidly. These systems are testable by 80-96 hours post fertilisation. Therefore, a significant proportion of our experimentation can be performed using early stages of development prior to them becoming protected under ASPA 1986. Additional factors that make it a desirable animal model include ease of

genetic manipulation and transparency of embryos which permits visualization of deep structure within the animal.

Typically, what will be done to an animal used in your project?

We will use genetic techniques (such as CRISPR-Cas9 mutagenesis and morpholino injection) to decrease the expression of genes and identify whether it resembles the human disease, infantile nystagmus. To make genetic changes in zebrafish we will inject molecules and proteins into the zebrafish embryo which will target the exact gene of interest we have identified to be linked with human infantile nystagmus. Microinjections are performed at the 1-cell stage (within a few minutes of fertilisation) and take less than a few seconds per embryo. After the visual system has developed, we will record the eye movements of zebrafish in different conditions and using rotating black and white stripes of varying widths to determine visual acuity. Similarly, we will test the swimming behaviour using the same black and white stripes to see if the fish can follow the movement of the stripes. These behavioural assays are considered mild severity as they are natural reflex response that are part of the developing visual system. The overall duration of these tests are brief typically lasting less than 1 hour. We will record how the retina functions using a technique called electrophysiology in response to various light intensities. We will characterise the structural retinal changes using non-invasive imaging techniques which are used in humans. We will introduce drugs to improve eye movements with the aim of correcting the developmental errors arising due to the genetic changes. This would be manifested as an improvement in vision after drug treatment.

What are the expected impacts and/or adverse effects for the animals during your project?

The microinjections will be performed in the 1-cell stage. At this stage there is possibility of damage to the embryo and non-viability which will be identified immediately or within the first 24-48 hours. The expected impacts from manifestations of the genetic changes include abnormal eye movements or reduced vision. This has previously been documented in the belladonna and albino mutant zebrafish. This would be similar to patients with nystagmus, abnormal eye movements such as nystagmus can be a sign of poor vision and can be present from larval stages. For the drug assays, we will utilise data from previous dose response studies and unpublished data from collaborators to minimise adverse effects. Moreover, we will first test the drugs at lowest concentrations in zebrafish larval stages with incremental increases in drug concentration to achieve the therapeutic response. This will ensure that we maintain mild severity for majority of animals for the main experiments. Most drugs that have been previously tested and thought to act on the nervous system. We will assess zebrafish larva for endpoints that include a loss of balance, or changes in locomotion or breathing. If we observe any change in behaviour we will humanely cull the animal the experiment and contact the NVS or NACWO for advice before proceeding further.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild severity for protocols 1, 2, 3 for 100% of animals

Non-recovery for protocols 4, 5

For protocol 6 mild severity for 90% and moderate severity for 10%.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Currently there are no curative treatments available for infantile nystagmus. This is partly due to a lack of a suitable validated pre-clinical model to test drugs. Zebrafish are ideal for our experimental design since their visual system develops rapidly; the systems that control eye movement and vision are functional and experimentally testable by 5 days post fertilization (5 dpf). The eye movement and vision responses continue to develop beyond 5 dpf. The systems that control eye movements are similar to those observed in humans. Previous literature have used mice as an animal model for infantile nystagmus with limited success. We will use zebrafish as an animal model for infantile nystagmus and this represents a replacement of previous approaches by using a species at an early development stage. Unlike other animal models, such as mice, zebrafish are more suited for the scientific hypotheses we plan to test. This is due to the similarity between the visual systems of humans and zebrafish. Cones and rods are the light sensing receptors at the back of the eye in the retina responsible for fine and gross vision respectively. Zebrafish are cone dominant making them ideal to study the visual system unlike mice and rats which are rod dominant.

Which non-animal alternatives did you consider for use in this project?

It is unlikely that we can reproduce all the neural circuits needed to control eye movement in a cell or tissue culture, making it necessary to use animals in these experiments. We have checked the FRAME website, NC3Rs website and other sources for other ways to address this question and have not identified a suitable alternative.

Why were they not suitable?

Computational and cell culture studies are not sufficiently advanced to replicate the complexities of oculomotor development, additionally it would not provide sufficient phenotype data to translate into human clinical trials. Moreover, safety and toxicity data for drugs are more translational to human trials from an animal model rather than a cell culture assay.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the sample size based on previous published experiments and the number of anticipated genotypes. We utilized a dedicated sample size calculation software (StatMate for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com) to perform the power and sample size calculations. We may also consider using NC3R EDA during further experimentation.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have discussed our experimental plans in collaboration with research groups with extensive experience in eye movement analysis in zebrafish. Their datasets and previous publications were utilized for our sample size calculation. We utilized a dedicated sample size calculation software to perform the power and sample size calculations.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where genotypes of interest are already available, we will source them from the Zebrafish International Resource Center (ZIRC) or other centres with authority to provide them for use. Having an established line in another laboratory and utilizsing this in collaboration is more advantageous and reduces duplication. To this end, we have approached collaborators and agreed to share resources (mutant lines) to ensure number of fish used are kept at a minimum. We will adjust breeding and maintenance numbers and tailor mating to ensure we have just enough offspring per experiment.

Similarly, our sample size calculations are robust and based on strong pilot and previous experimental data. We will also make our raw data available so that it can be re-analysed by other research groups.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use zebrafish as an animal model. We will use fish prior to independent feeding for most experiments thereby limiting the number of animals used under regulated procedures. In some cases, we may measure the behaviour of older fish, such as 7 dpf because they display a more robust nystagmus response and optomotor response.

Why can’t you use animals that are less sentient?

To generate the nystagmus phenotype entire neural circuits in the brain and retina are required. Similarly, to test the impact of drugs and toxicity profile the whole fish is required. The zebrafish that we will use will be of lower sentience than mice and since our experiments use a very early stage in development. Therefore, these experiments are much less likely to cause any suffering. Healthy adult fish and knockout lines will be maintained at a minimal number to produce sufficient eggs for experiments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will utilize skin swabbing technique for DNA collection as suggested in the NC3R website (https://www.nc3rs.org.uk/skin-swabbing-dna-sampling-zebrafish).This is considered potentially less invasive compared to the traditional fin clipping technique. Thus, this represents a refinement for DNA sampling.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will utilize well established protocols and where possible collaborate with labs where these protocols have been developed. This will ensure there is minimal harm and distress for the fish. Moreover, our facility has extensive experience in breeding and maintaining transgenic zebrafish. This work will also be performed in collaboration with labs where the swab-based DNA sampling technique was developed. This is a significant refinement to traditional DNA sampling techniques.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Regular NC3R newsletters and other resources regarding advances in 3R’s are circulated by the deputy director of our preclinical research facility and named information officer (NIO). Moreover, regular attendance of meetings organized by the division of biomedical sciences will help keep us informed and subsequently implement changes to reflect advances in 3Rs.

On completion of each experiment, we will review raw data and protocols to determine any refinements that can be applied to future experiments. Additionally, we will be collaborating with other centres interested in this work and therefore by sharing the results and experiences with methodologies will allow refinement of techniques.

77. Convection Enhanced Delivery for the Treatment of Disease of the Brain

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

convection, brain, cancer, CED, dementia

Animal types	Life stages
Sheep	adult
Pigs	juvenile, adult
Minipigs	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To facilitate the testing of developments in Convection Enhanced Delivery (CED) systems and to evaluation the safety and efficacy of drugs administered using this technology for the treatment of neurological diseases, including brain tumours.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Diseases of the brain are a major cause of suffering and death, and the lack of effective treatments constitute a largely unmet medical need. In the UK, an estimated 16,000 people per year will be diagnosed with a brain tumour, the number one cause of cancer deaths in children and young people. Over 850,000 suffer from dementia, more than 600,000 have epilepsy, around 6,700 have Huntington's disease, and about 5,000 have motor-neurone disease. Currently there are no curative treatments for any of these conditions.

A major obstacle to the treatment of diseases of the brain is the difficulty in achieving therapeutic levels of drugs at the tissue level due to the low permeability of blood vessels within the brain which effectively prevent the passage of all but the smallest of molecules, the so-called blood brain barrier. As a consequence, the use of drug for the treatment of brain cancers and neurodegenerative conditions has been severely restricted. To circumvent the limitation to drug delivery, posed by the blood brain barrier, my group has pioneered the development and use of Convection Enhanced Delivery (CED).

This system utilises catheters, inserted into the target region of the brain, to infuse the drug at a low flow rate under pressure. As a consequence, the normal distribution of the drug is enhance by the resulting pressure gradient created within the tissue enabling therapeutic levels to be obtained at the target site. In order to ensure that the insertion path of each catheter avoids blood vessels and vital structures an analysis of a 3D image of the brain, obtained by high resolution MRI scanning, is undertaken. At the time of scanning an external MRI compatible head frame is fitted to the animal to provide external reference points to enable the precise insertion point and trajectory for each catheter to be determined, using either a manual or robotic device.

As a result of the work conducted thus far CED has enter clinical use within the NHS however, further development work is needed to improve the technology that underpins this delivery system in order to optimise its effectiveness and exploit its full clinical potential. In addition, there remains a need to demonstrate the safety and efficacy of therapeutic agents delivered using this system and to obtain the data needed to progress such treatments into human clinical trials. The purpose of the outlined studies is to facilitate these developments in order to realise the full potential of CED for the treatment of diseases of the brain.

What outputs do you think you will see at the end of this project?

The primary outputs from this project will be the efficacy and safety data needed to progress treatments given by Convection Enhanced Delivery towards human clinical trials. These outputs will include data on drug distribution, neuro- and systemic toxicity, imaging characteristics and infusion methodology.

Who or what will benefit from these outputs, and how?

The primary benefit of these outputs will be the advancement of novel treatments for diseases of the brain towards human clinical trials. In the short term, the information gained will be of benefit to scientist and engineers working to optimise Convection Enhanced Delivery systems. In the medium term, the work is expected to facilitate the translation of novel treatments into human clinical trials. In the long term, it is to be expected that the work undertaken will lead to the introduction of more effective treatments for disease of the brain into clinical practice.

How will you look to maximise the outputs of this work?

The findings of the study will be disseminated through presentations at scientific conferences and publications in peer reviewed journals, whenever this is not precluded by intellectual property rights. I engage extensively in collaborative projects, which provide the opportunity to both gain expertise and disseminate knowledge. I have a proven track record in the publication of research findings from work undertaken in previous projects and will continue to do so wherever this is possible.

Species and numbers of animals expected to be used

- Sheep: 120
- Pigs: 120
- Minipigs: 70

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In order to generate the safety and efficacy data needed to progress the translation of novel treatments towards human clinical trials, it is necessary to use an animal model that closely replicates the conditions applicable to humans. For the outlined studies, the key criteria for the animal models is a brain that is similar both anatomical and in size to that of human. Consequently, sheep and pigs have been selected for these studies as their brain closely resembles that of human's anatomically and, although smaller, is of sufficient size to enable comparable volumes of drugs to be delivered to target regions. Juvenile pigs will be used for studies involving only a single treatment. Mature adult sheep will be used for long term or repeat delivery studies as these require animals that are no longer growing.

Typically, what will be done to an animal used in your project?

Animals will undergo a surgical procedure that closely mimics that used in human clinical practice. A surgical plane of general anaesthesia will be induced, and the animal fitted temporarily with the head frame used to align the Convection Enhanced Delivery (CED)

catheter. Brain imaging will be undertaken to determine the coordinates for catheter insertion. A small skin incision will be made at the catheter insertion point and the underlying skull exposed. A small burr hole will be drilled through the skull and the CED catheter inserted to the required coordinates. The drug will be infused, and further imaging may be undertaken to assess its distribution. For pigs, the catheter will be withdrawn whilst for sheep it will be fixed to the skull. The head frame will be removed, and the skin incision closed, in the case of sheep leaving the catheter end exposed (capped). The animal will be recovered from anaesthesia and post-operative analgesics given until it is free of pain.

Sheep may undergo one or more additional infusions, under general anaesthesia, or may have the catheter attached permanently to a delivery device implanted at the time of surgery.

At predetermined time points, animals will be re-anaesthetised and killed by anaesthetic overdose so that tissues can be collected for subsequent analysis to determine drug distribution, drug tissue concentration and to assess safety by histopathological examination.

What are the expected impacts and/or adverse effects for the animals during your project?

Before any procedure are undertaken, the animals will be habituated to human contact for at least a week to minimise any stress whilst inducing anaesthesia or when undertaking post-operative checks. During anaesthetic induction, animals may experience mild transient pain caused by the insertion of a hypodermic needle. Thereafter, animals will be maintained at an anaesthetic plane that ensures they do not incur any suffering. All animals are expected to make an uneventful recovery from surgery and the analgesic regime used will ensure that any pain is minimised. All animals are expected to resume normal behaviour within a few hours of recovery. The drugs used are not expected to result in any harm to the animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Sheep- Moderate (100%)
- Pigs- Moderate (100%)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To progress novel technological developments and treatments into clinical practice, it is essential to demonstrate their safety and efficacy using representative animal models that meet the requirements of the regulators responsible for authorising first in human clinical trials.

Which non-animal alternatives did you consider for use in this project?

At all stages of the work, we seek to use non-animal alternatives and only progress to assessments involving the use of animals where there is no alternative to obtaining the data required. To this end we have developed and optimised gel/phantom models for preliminary assessment of CED catheters and the distribution of drugs, which will be used and further developed during the course of the outlined work

Why were they not suitable?

We have developed non-animal alternatives for preliminary assessment of new technologies and novel agent that ensure that only those that are likely to succeed are progressed into animal studies.

However, as yet, the data generated using these does not meet the requirements of the regulators responsible for authorising progression to human clinical trials. Consequently, there is no alternative to the use of animals for the outlined studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of animals required for the outlined studies has been determined using data generated during similar work conducted under my previous licence to determine experimental groups size; the pipeline of work already secured and the use of the NC3Rs Experimental Design Assist application to ensure the appropriateness of the study design. Statistical input is sought, where necessary, to strengthen the overall scientific quality and relevance of the studies to be performed, with sample size calculations performed for specific studies to determine the group size.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Power calculations were undertaken to determine experimental group sizes, using data generated during similar work conducted under my previous licence. Where appropriate, control group data obtained in previous studies will be used to minimise or eliminate the need for specific controls. I utilise the experimental design software, such as that NC3Rs Experimental Design Assistant when planning experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where appropriate, control group data obtained in previous studies will be used to minimise or eliminate the need for specific controls.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The outlined work aims to generate safety and efficacy data to support the translation of new treatments for diseases of the brain administered using Convection Enhanced Delivery. The procedures used closely mimic established techniques use clinically within the NHS. All procedures will be conducted under general anaesthesia and upon recovery an analgesics regime will be implemented that ensures pain is minimised. All animals are expected to recover uneventfully from the procedure and to resume normal behaviour within a few hours. No adverse effects are expected as a result of the drugs administered.

Why can’t you use animals that are less sentient?

To undertake the outlined study, an animal with a brain of similar anatomical structure and size to that of humans is required. Only highly evolved species of a moderate size, such as the pig and sheep, are able to meet these criteria.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The outlined procedures have been refined during work conducted under my previous licences. Upon arrival in the unit, the animals will be habituated to human contact before

any procedures are undertaken. Surgery will be conducted in a manner that mimics that used clinically within the NHS. Following surgery, the animals will be provided with post-operative pain control until they show no discernible signs of pain. All animals are expected to resume normal behaviour within a few hours of recovery from anaesthesia and to continue to live normally throughout the study. I am committed to ensuring that all of the procedures undertaken by my group are refined to minimise suffering and that the experiments are designed to use the least number of animals needed to obtain the required data.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The procedures used will closely match those used clinically within the NHS and will be conducted using full aseptic precautions in line with LASA guidelines for aseptic surgery. Studies will be conducted in compliance with GLP standards.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

My institute places a strong emphasis on the promotion of the 3Rs and organises regular event to raise awareness and encourage the dissemination and uptake of 3Rs developments. We are supported by a regional NC3Rs representative, who actively publicises and promotes engagement with the 3Rs. The institution's AWERB committee promote the 3Rs by challenging licensees to demonstrate their full commitment during licence reviews and the NVS and NACWO review all pre-study briefings and raise any 3Rs concerns with the applicant when these arise.

78. Testing Novel Strategies for Bone Repair in Sheep

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Skeletal, Tissue engineering, Biomaterials, Stem cells, Bone repair

Animal types	Life stages
Sheep	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aims of the project are to develop and test biomaterials that can repair broken or diseased bone. Our rationale is centred on the urgent need to develop materials capable of activating and growing both bone and blood vessels to help patients repair their damaged and diseased skeletal tissue.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

With an increasing ageing population, skeletal tissue loss due to injury or disease is rapidly growing. This significantly impacts the quality of life for the patient over time and has socio-economic costs for healthcare providers. For example, each year in the UK there are over 50,000 primary hip replacement operations at a cost in excess of £350million. Hence,

for reconstructive bone surgery and fracture repair, the need to develop better techniques and alternative bone therapies is vitally important.

From a patient's perspective, the ultimate goal is to repair and replace their damaged skeleton with bone material, harvested with minimal complications from their own skeleton. However, the current clinical approach for the treatment of bone defects and non-unions is bone grafting or metal implants, which both have significant drawbacks. The combination of a bone stimulating material combined with bone tissue generating cells to produce a therapy would be an invaluable surgical option, reducing both complication rates occurring in patients undergoing bone replacement surgery. Therefore, generating materials that can help bones repair themselves will be invaluable for patients and animals suffering from diseases and fractures affecting the skeleton. These include non-healing bone fractures and weakened bones due to diseases like osteoporosis and bone cancers.

What outputs do you think you will see at the end of this project?

The proposed models being used in these studies will help us to gain new insights in the development of tissue regenerating biomaterials to rebuild broken bones

Specifically.

We want to understand how biomaterials stimulate bone cells and tissue to grow and repair large bone fractures.

We hope to develop innovative biomaterial structures that can stimulate bone regeneration in large bone defects in order to provide new and improved treatments for patients who have fractured bones or who have lost bone owing to other diseases like cancer.

We will present our findings at scientific and medical conferences and we will publish these outcomes in peer-reviewed scientific journals.

Finally, any new procedures/methodologies for improved welfare settings developed during the project licence will be published and shared with the scientific community in order to benefit both human and animal patients.

Who or what will benefit from these outputs, and how?

As with all our research goals the aim is to develop improved therapeutic strategies and products for patients who suffer from skeletal diseases and bone trauma injuries. We envisage that new biomaterials have the potential to provide improved treatments to help rebuild bones and skeletal tissues. In addition, the findings from musculoskeletal research can be applied to the veterinary field due to similarities in orthopaedic conditions between people and animals, leading to novel applications in a ‘one health’ approach.

Beneficiaries will include:

Patients and animals suffering from broken bones and skeletal diseases. Healthcare providers.

UK, EU and worldwide tissue engineering/biotechnology companies involved in tissue regeneration, stem cell biology or developing innovative tissue scaffold technologies,

The academic community in the generation of new protocols and avenues for skeletal tissue regenerative research.

Many materials we use in our studies are biocompatible and currently used in clinical practice for other applications. Modifying them to enhance their properties can result in new therapies in a relatively short time frame, benefitting patients due to the known track record of efficacy and safety. However, with the complex materials proposed the final outcomes for patients may require months to years of investigation due to thorough testing to ensure safety and efficacy prior to use in the clinic.

When these new treatments become available, we envisage growing the tissue constructs in the laboratory and transferring the regenerated samples to the patient in theatre. Ultimately, we believe this work will be translated to the clinic and benefit patients within the National Health Service and the wider medical community in the area of musculoskeletal repair. Alternatively, the development of materials that exploit the regenerative potential of the patient's own repairing cells would minimise costs and hasten the material's therapeutic implementation.

In addition, the information from this project, including the study protocols and techniques will be made freely available via publication in peer-reviewed journals, in order to benefit patients, other researchers, doctors, vets, and pharmaceutical companies who are involved in the development and assessment of novel therapeutics which target the repair and regeneration of skeletal tissues due to disease and injury.

How will you look to maximise the outputs of this work?

Training & Collaborations

The team undertaking the research will acquire skills and expertise in bone repair, blood vessel and skeletal stem cell biology, biomaterial development, imaging techniques in real-time such as 3D x-ray scanning and 3D optical imaging. Training will be achieved through an extensive local network of multidisciplinary collaborations within the University Hospital and established national and international collaborations in regenerative medicine.

In addition, the protocols and research work will be communicated to our student & postdoctoral researchers to provide information regarding the models available for research projects at the University and to foster collaborations. We are part of the Regenerative Medicine Platform collaborative group network where we share our methods and results from acellular materials that will be available to be tested in skeletal and other tissue/organ regenerative models.

Education & Public engagement

The information and findings generated from this project will be presented through the scientific community by presentations at national and international meetings. We will also communicate findings to the public through outreach activities such as the University Science Day, and laboratory open days for GCSE and A-level students in tissue regenerative medicine.

Progress of the study and results will be regularly presented at ongoing teaching events and public lectures. The press office of the University will also publicise the results.

Species and numbers of animals expected to be used

- Sheep: 48

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Whilst all attempts are made to reduce the use of animals by using cell-based laboratory methods, it is inevitable in work of this nature that animal investigations have to be undertaken. We can determine a number of key factors to ensure the biomaterials we make is suitable for bone repair. However, there are also a number of biological factors (angiogenesis, inflammation, multiple stem cell recruitment, proliferation, differentiation in a coordinated manner. Multiple coordinated secretion of cytokines and growth factors to stimulate bone) that we are still unable to recapitulate in the lab that only a living organism can provide.

Biomaterials will be examined for the bone repair in smaller animals such as mice or rats. Only the best bone repairing biomaterials will be considered for scale up and use in repair of large critical sized bone defects.

Bone disease and fractures may occur across the lifespan, ranging from young to old, however, it is predominantly in the adult and aged populations that the repair mechanisms in bone start to fail.

Therefore, it is expected that the vast majority of experiments will be performed in adult animals.

One of the biggest hurdles to overcome in clinical orthopaedic repair is the regeneration of large segments of bone that have been lost due to disease or trauma. We will therefore use skeletally mature female sheep (as they have a similar body size and rate of bone remodelling as humans) in these studies as this provides a larger bone defect model to

test the potential of our repair materials to regenerate significant amounts of bone tissue before it can be translated into use in a humans.

Typically, what will be done to an animal used in your project?

Sheep through a surgical procedure will have a section of bone (maximum width of 11 mm x 15 mm height) removed from the femoral condyle of lateral hind limbs. As each leg serves as a separate sample, the number of animals used in the study is reduced. Biomaterials will be implanted into the bone defect site, with an empty defect acting as negative control and implanted bone grafts acting as a positive control (gold standard of bone current bone repair). Bone trephines have been designed to allow sufficient bone cores to be collected from two femoral condyles in a single sheep to fill one defect with bone graft, therefore removing the need for bone harvesting from the iliac crest. An empty defect is used as the negative control. Pain relief will be administered during and after the operation. Sheep will be slightly lame for up to three days but are able to stand and move directly after surgery with no hinderance in feeding and drinking.

The animals will be housed indoors in pens for the duration of the study which further activity (e.g. Fitbark) and clinical monitoring may be undertaken. The duration of the study will be for up to 52 weeks

In addition to the above scenarios

1. the animals may receive on several occasions an injection of bone inducing factors to localise to the bone defects to enhance the repair.

2. The animals may receive on several occasions an injection of bone labelling drugs e.g. tetracycline to label new bone formation

3. The animals may be subjected to a scanning device, such as ultrasound, in order to improve the implanted biomaterials' ability to promote bone healing.

4. The animals may be subjected to imaging of the defect site by radiography, CT, ultrasound as deemed appropriate to visualise the defect and assess healing at different time points e.g. on the day of surgery, week 4, week 8, at the study end etc.

At the end of the study, animals will be killed and when dead the femoral condyles will be removed and fixed for further analysis. Defects will then be scanned using micro-computed tomography (micro-CT) before carrying out histology and immunohistochemistry to assess bone quality and cell types.

What are the expected impacts and/or adverse effects for the animals during your project?

In this model the overall severity the animal will experience is moderate short term pain due to the procedure. With the limb defects we will expect the animals to be slightly lame

for a couple of days with no adverse effects from the surgical procedure. Normal movement and locomotion resumes thereafter. In our experience the sheep are standing within 10-15 mins post recovery after surgery. Analgesia will be continued for 72 hours (and continued if required after veterinary consultations) after recovery and closely monitored during this period.

Although the animals will be regularly monitored, weight loss has not been an issue with these procedures.

X-ray scanning. There is the risk that radiation doses during an x-ray examination may produce adverse effects such as delayed growth or repair. However, doses of x-rays and duration of scans are way below any threshold that will cause problems to the animal. These scans are usually in the duration of 2- 5 minutes.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For the bone defect models in these protocols, we estimate the severity to be moderate and that 100% of the animals will experience this due to the surgical procedure.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Initial bone tissue engineering research work and biocompatibility will utilise all relevant laboratory cell based methods at our disposal. However, the clinical relevance of the approaches proposed, and the quality and quantity of bone tissue formed can only be assessed in relevant animal models. Cell growth, toxicity measurements and bone forming biomaterial assessments performed in the laboratory cannot, to date, mimic the complex physiological in vivo microenvironment involved in bone tissue repair. Thus, this proposal necessitates the use of animals to test the ability to generate new bone using the bone tissue engineering principles outlined. It is important to stress that initial laboratory cell based studies are undertaken to evaluate and optimise the growth of human bone cells and skeletal stem cell populations on the scaffolds under examination. Where possible we will utilise our organotypic/organ culture ex vivo models as a replacement to the animal models to examine the ability of our regenerative strategies to repair skeletal defects.

Finally, to address the efficacy of biomaterials in the repair of large bone defects there requires an understanding of its effects on integration, inflammation and blood vessel formation, that can only be adequately examined in the environment of the whole animal.

Which non-animal alternatives did you consider for use in this project?

Using laboratory cell culture experiments we are gaining as much information as possible to understand the biocompatibility, functions and toxicities of new biomaterials being developed. In addition, we are using 3-dimensional bone tissue known as organoids to study the interaction of the many cell types that create fully functional bone tissue. We are using this data to create biomaterials with functioning coatings which can mimic the bone growth processes.

In other studies conducted in our group Artificial Intelligence (AI) modelling is being developed to test biomaterials implanted into the developing chick membrane, so that this technique can be used to predict and determine how a new material will act in producing blood vessels and new bone tissue.

However, there are limitations at the moment as this AI modelling is based on data provided from real studies. Once enough data has been accumulated for AI, this can, in principle, be applied to the animal models used in these protocols when researching new biomaterials. As AI modelling obtains a better understanding of the outcomes and mechanisms of these materials in the aforementioned bone models, the number of animals required will eventually decrease. With continual learning of the models and how new therapies interact in repairing and rebuilding bone AI in the future will be one avenue to replace the use of animal models.

Previous human studies have been reviewed for evidence-based information and outcomes. There is a wealth of clinical literature providing data on the efficacy of biomaterials on implants in hip replacements that will provide us with guidelines of what works in the patient. In addition, there are number of clinical trials with data on the dosing of growth factors and of skeletal stem cells which give insights into the positive outcomes and the negative outcomes. We will use this information to improve our biomaterials and avoid the negative consequences that have already been discovered in these clinical trials.

Why were they not suitable?

The above strategies are suitable for screening, in part, the many biomaterials that are developed. We only resort to animal studies due to the complex factors that are involved in regeneration and repairing of bones. The generation of new bone requires multiple steps and the interplay of many different bone factors (inflammation, skeletal stem cells, blood vessel cells, collagen structures, calcium and phosphate (for hardening the new tissue), mechanical stimulation and cells that re-shape the new bone in a 3D environment to fit the defect bone exactly (without scarring) in a co-ordinated manner.

This interaction cannot be consistently or correctly reproduced under tissue culture conditions to meet the criteria expected to inform a clinical translation. Furthermore, in vitro model systems make it difficult to detect unexpected toxicities or inflammatory responses, as well as observe material integration and degradation over time.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used our current and past experimental data to inform us of an estimation of the number of animals required per group and the number of control and test groups to give statistically valid experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In the design phase we have used organ culture and chick eggs to reduce intra-group variability of biomaterials and by using this model and the results obtained has allowed us to reduce progressive experiments in mice models. This screening has reduced approximately 60% the regenerative biomaterials being taken forward for testing in animal studies.

In addition, we have run experiments in mice models to determine the suitability of scaling up to be used in a large sized bone defect model in the sheep. Only the biomaterials with significant bone repair and biocompatibility shown in the smaller animal models are considered for translation to the large sheep model.

We have designed experiments using the fewest animals consistent with obtaining statistically valid results as determined from our power calculations. Using the NC3R's Experimental Design Assistant we have calculated the minimum number of animals required for a determined amount of new bone tissue formation in the test implant material comparison to control (no implants) or control (implanted biomaterials without stimulatory factors). From previous studies we will be using a minimum of 6 defects per experimental group (i.e. 3 sheep) to attain significance between the test groups. Where possible we will use historical data obtained from previous studies to compare negative and positive controls so long as the time points, dimensions and sheep age and breed remain the same.

In certain cases, mathematical modelling may be used to predict the release of growth factors from biomaterials under certain biological conditions, allowing us to determine how many animals we can exclude while still achieving a meaningful outcome in the experiments. In addition, multiple scanning and imaging of the animal bone implant or bone defect site at different time points reduces the number of animals required in these types of studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Rigorous laboratory testing will be performed on the newly constructed biomaterials before any candidates are assessed in the rodent models. Only after assessment in these models with a (stop/go scenario) will the optimal biomaterial candidates be considered for scale up and testing in the sheep bone defect model. Based on previous studies from our group and collaborations both nationally and internationally we have collected historical data of control autograft and empty defect bone repair results where we can use less animals for the control groups.

Multiple analysis will be undertaken on biomaterial samples implanted into the animals, this will include X-ray and microscope imaging, blood vessel scoring, biochemical and molecular analysis, and histology. The multiple data that can be derived from one sample will optimise and minimise the number of animals to be used in a single study. Finally, at the end of the study we collect marrow from the iliac crest for isolation and storing of sheep stem cells and in addition, any unwanted tissue may be harvested by other groups for us in other and further research projects.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The disadvantage of using small animal bone defect models, such as mice, is the intrinsic disparities in bone composition, density, and quality between humans and small animals. These models are not suitable to evaluate at scale materials for bone repair for the translation to the clinic. For this reason, we are employing a larger species, sheep, which closely resemble the bone structure, mechanics, and repair characteristics associated with humans. Sheep have been used in numerous bone conditions to assess the benefits of biomaterials to regenerate bone. The femoral condyle defect model causes the least pain,

suffering and complications as complex support structures to hold the bone defect in place are not required reducing the chances of implant failure, additional bone fractures and infection that can occur with other bone defect models.

Why can’t you use animals that are less sentient?

The development of new bone is incredibly complex involving factors and cells from the blood, the bone marrow, inflammatory cells, minerals and mechanical forces. In addition, bone fractures take a considerable time to successfully repair.

Unfortunately, we are still not at a stage to incorporate all these components in the laboratory therefore these animal models are required to help us understand and improve the development of materials to aid in bone growth and repair. We can mimic some of the components in the laboratory, and this has led us to refine the materials and cells that we will use in these studies.

There is a chick egg embryo model that we use to determine the efficacy of these biomaterials in generating bone. This model is a less-invasive in vivo model allowing for the assessment and function of these biomaterials in a living organism. However, these are short term models (max. 10 days) whereas bone repair can take months to repair in vivo.

One of the clinical problems with bone defect repair is producing large amounts of material to implant in the defect. This cannot be assessed fully in less sentient animals. Therefore, the sheep bone defect model provides the size and the similar human bone physiology to test the potential of these biomaterials to repair large bone defects in humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We shall continue working to minimize welfare costs for the animals in the following ways:

Reviewing the need for these experiments to be undertaken. Weighing up the balance of potential successful outcome of undertaking the experiment and the level of harm/distress the animal will endure.

We will ask for feedback from technicians and welfare officers in the BRF and their valuable day- to-day knowledge to revise protocols to improve the animals' experience.

Prior to the surgical procedures, researchers will familiarise and handle the animals on a regular basis to reduce stress for both the animal and the user.

The femoral defect created in both hind limbs is well tolerated by the animal. This allows for 2 implants to be tested in the animal. This refinement has reduced the numbers of sheep required to carry out a study.

General best practise guidance for injection, blood sampling and aseptic techniques will be followed. This has been developed and refined as best practise over a number of studies carried out by the group.

Animals are closely monitored for several days after the bone defect surgery, with continued pain relief given for at least 72 hrs in the first instance and further pain relief administered if signs of pain persists in the animal. Wounds will be carefully monitored to ensure that sutures have not loosened or come off or there are any signs of infection.

The sheep will receive pain relief (a patch e.g. Fentanyl), as advised by the Named Veterinary Surgeon (NVS), pre- and post-operation to allow for a continuous supply of pain relief for an appropriate period. If for unforeseen circumstances the surgery must be cancelled the patches will be removed and the sheep returned to the pens. Sheep will not then be re-patched and starved for a minimum of 72 hours.

The sheep will receive local pain relief in the injection/incision site.

To reduce the risk of infection the sheep will receive antibiotics pre- and post-surgery.

There are three clinical score sheets to guide us when monitoring the animals. One covers the mobility, food intake and water consumption, body temperature, demeanour and respiratory rate and is routinely used for all animals for a minimum of 3 days post-operation. There is a posture and locomotion scale that is used to assess the levels of lameness and we use the grimace scale to assess levels of pain in the sheep.

Delivery of any material to a defect site will be closely monitored to ensure that there is not significant inflammation. If inflammation occurs the sheep will be carefully monitored and graded using a locomotion scoring system after consultation with the NVS. Any severe soft tissue inflammation will be treated with antibiotics and local pain relief provided to the animal. If this has not improved the animal's condition over 24 hours then the NVS will be consulted regarding the short term use of an anti-inflammatory drugs. If the sheep is significantly lame on both hind legs and is not responding to additional analgesia it will be killed immediately.

During a study, animals may be injected with fluorochromes, at 2 known time points post-operatively, to allow assessment of the rate of bone growth within the defect. The stain binds to calcium ions on the mineralisation front and stabilises after 24 – 36 hours after injection. This has been used in many animal studies with no adverse effects.

Animal weight is frequently monitored for the duration of the studies. In addition, an activity monitor may be worn by animals for either part or all of the duration of a study. The ‘Fitbits’ allow us to continuously record the ‘play’, ‘rest’ and ‘activity’ of the sheep before and during the experiment. This allows us to identify early indicators of reduced welfare in the animals and to intervene accordingly. If reduced activity or play and increased rest is

noted, the NVS will be asked to examine the animals to identify the cause of the reduced mobility.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the ARRIVE guidelines which provide a 20-part checklist of the minimum information required to be reported by groups using animals in research. ARRIVE guidelines are essential to help overcome issues in science such as reproducibility, reducing bias and the correct use of statistical methods of analysis.

In addition, we will follow and consult the Norecopa (Norway's National Consensus Platform for the advancement of "the 3 Rs" (Replacement, Reduction, Refinement)) in connection with animal experiments database platform and PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for better science experiments using animals to ensure that we are using the best defined models for our work in the investigations of biomaterials for skeletal biology.

All surgery will be carried out aseptically in dedicated facilities, according to the LASA guiding principles for preparing for and undertaking aseptic surgery.

Our group will meet on a regular basis to discuss best practises (new publications, surgical methodology) for upcoming studies, as well as post-study analysis to improve experimental design and animal welfare for future studies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will consult the NC3Rs website to identify any changes that will be relevant to the workings of this model. In addition, we will follow the latest findings from the Laboratory Animal Science Association

(LASA) and the PREPARE guidelines from Norecopa in better planning for research involving animals to prepare for better science and advance the 3Rs. Any changes will be implemented directly through the experimental design, and if necessary, through a project licence amendment.

In addition, we follow the latest publications on using these models and identify any new methods that reduce, replace or refine the biomaterial implant and bone defect models. If applicable to this model in improving the 3R's we will request amendments from the Home Office to adjust the techniques/methods required and training or notification of relevant staff in updated techniques.

Continual training will be undertaken to ensure that users will be proficient in running these studies particularly on the aftercare post- surgery.

Attendance at BRF meetings and/or discussion of BRF meeting slides at group lab meetings.

We will liaise with other experienced research groups, clinicians and veterinarians who have experience of running these models to obtain advice on updates on best practise for anaesthesia/analgesia, husbandry and surgical procedures similar to the ones we will be undertaking in this project.

79. New Tools for Improving Ovarian Cancer Care

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:

(i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Ovarian cancer, STIC lesions, Sperm, Spermbots, Cellular microrobots

Animal types	Life stages
Mice	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To engineer sperm as novel diagnostic and therapeutic tools to sense and eradicate early ovarian cancer lesions arising inside the fallopian tube.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Ovarian cancer ranks fifth amongst cancer deaths in women and top amidst all gynaecological cancers. Due to a lack of early detection and treatment methods, the field has long been hampered by low survival rates (<30% at ten years) and has seen few advances in ovarian cancer management for decades. Indeed, apart from surgery, conventional cancer chemotherapies remain the backbone of current therapies. These generic treatments elicit severe side effects, highlighting the sore need for new and more specific approaches, such as the ones proposed in this project. Ovarian cancer has long been thought to arise inside the ovaries. Instead, it is now well established that the most common and aggressive ovarian cancers predominantly develop inside the fallopian tube as so-called serous tubal intraepithelial carcinoma lesions (STICs) before spreading to the ovaries and beyond. This paradigm shift opens up an unprecedented opportunity for detecting and treating ovarian cancer earlier inside the fallopian tubes, such as via the engineered 'spermbots' to be developed in this project.

What outputs do you think you will see at the end of this project?

By the end of the project we will have shed light on currently under-studied early ovarian cancer characteristics, as well as tested the potential of engineered sperm as novel diagnostic and therapeutic tools in relevant in vitro and in vivo settings.

Who or what will benefit from these outputs, and how?

Combined with the patient and public involvement and engagement we will perform (e.g. taking advantage of regularly meeting ovarian cancer patient groups at the Establishment), this work will form the groundwork towards future clinical translation. In the longer term, our studies could serve as a paradigm for a new platform of cell-based diagnostic and therapeutic treatment agents, highlighting the ground-breaking nature of the work. Thus, if successful, our findings have potential to, in the longer term, lead to a radical change in practice, in which engineered human sperm would be used routinely for diagnostic and prophylactic purposes. In particular the approach might be used in women who are most at risk of developing ovarian cancer: postmenopausal women and women with certain genetic predispositions such as mutations in the breast cancer (BRCA) genes. As such, this proposal has potential to overcome major hurdles in the field that have prevented significant improvements in these areas for decades.

How will you look to maximise the outputs of this work?

Our findings will be made available to other scientists through collaborations, publication in peer- reviewed journals and presentations at scientific conferences and meetings.

Species and numbers of animals expected to be used

- Mice: 420

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is a model organism most closely resembling humans. The human and mouse genomes are approximately the same size, and display an equivalent number of genes, which are functionally conserved. Mice can be genetically altered, there is extensive literature concerning the topics of our investigation, and our own studies can be enhanced by exploiting appropriate ovarian cancer models developed by others in the field. These mouse models will be important for placing the findings of our in vitro (test tube) studies into an appropriate and meaningful in vivo (living organism) context. It is the combination of in vitro and in vivo studies that will provide the insights required to understand the early ovarian cancer events occurring inside the fallopian tubes and subsequently exploit these findings to the development of new theranostic (diagnostic and therapeutic) approaches, such as the engineered spermbots to be developed in this project.

Typically, what will be done to an animal used in your project?

Events mimicking early ovarian cancer developments occurring in humans will be induced by different means: (1) Female mice will have genes, known to be key in the generation of ovarian cancer, modified, so that they can be switched on by a chemical inducing agent when added for example to the drinking water or food of mice; (2) We will also undertake studies, initially pilot (using very small numbers of mice), to determine if ovarian cancer cells can be directly injected into the oviduct or neighbouring lower reproductive system sites, e.g. under ultrasound imaging conditions and recovery anaesthesia. At various time points after this the mice will then be inseminated, with sperm engineered to sense and/or detect early ovarian cancer lesions or appropriate control treatments (e.g. unmodified sperm and individual components added to the sperm during their engineering process), so that the various modalities can reach the oviduct. Towards this, we will perform pilot studies to optimise the least invasive method of applying sperm to the mouse reproductive system, so that they can reach the oviduct, such as artificial insemination via the vagina requiring no anaesthesia or injection into the uterus or oviduct, imaging-guided or via surgical means under recovery anaesthesia. The ovarian cancer development and efficiency of the engineered spermbots in targeting and eradicating early ovarian cancer lesions will be monitored by a combination of palpation and non-invasive imaging (the latter under recovery anaesthesia). Targeting of the sperm towards early ovarian cancer lesions will be tested by coupling the sperm to ovarian cancer cell-targeted antibodies and/or other targeting moieties, such as magnetic caps to facilitate guidance via external magnetic fields, which we will trial in pilot experiments. The success of these approaches will be compared to current standard-of-care treatments for ovarian cancer (such as doxorubicin-/platinum-/paclitaxel-/olaparib-based chemotherapies) using the least invasive method. Occasionally, the mice may be perfused, once only, with preserving agent whilst

under non-recovery anaesthesia. At the end of the study the mice will be killed humanely and tissue taken for microscopic examination.

What are the expected impacts and/or adverse effects for the animals during your project?

See section below.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The most severe of procedures will be the the induction of cancer lesions/tumours in genetically engineered and normal mice and insemination combined with regular induction of anaesthesia. All of the mice used will experience this since it is required for the insemination/injection of the mice and the guidance/imaging procedures. For Protocols 2 and 3 the most severe procedures will be the above combined with the induction of cancer lesions/tumours in genetically engineered and normal mice. The majority of mice (up to 95%) are not expected to show signs of adverse effects that impact on their general well-being. In line with this, the STIC lesions induced in the mice will not cause adverse effects per se, and the majority of mice will not progress to tumour development as a consequence of the spreading of STIC lesions to the ovaries and beyond. The vast majority of the procedures will therefore result in no more than transient discomfort associated with any of the individual steps and no lasting harm. The health of all mice will be observed daily. Notwithstanding this, all the mice will be humanely culled at the end point of the experiments.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to have a species with an intact reproductive system, including oviducts. Insects and fish are therefore unsuitable as they develop eggs ex vivo. Moreover, cancer development and spread involves a plethora of interactions between the cancerous cells and their surrounding environments, governed by multiple signals originating from both their immediate neighbours and distant tissues. Combined with the contributions that different components of the reproductive system have in facilitating sperm transport to the

oviduct, the proposed research cannot be carried out in tissue culture alone, but can only be addressed with the use of animals.

Which non-animal alternatives did you consider for use in this project?

We will fully take advantage of non-animal alternatives to test the engineered sperm in different 2- dimensional and 3-dimensional models of in vitro grown ovarian cancer cells. However, the aims of this study cannot fully be achieved without using living organisms, as there are too many factors contributing to sperm transport inside the female reproductive system and ovarian cancer development inside the fallopian tube to be appropriately modelled (see also above). However, extensively optimising the efficiency of different types of engineered spermbots in vitro, will allow us to take forward the minimal number of engineered spermbots for animal testing.

Why were they not suitable?

We need to have a species with an intact reproductive system including oviducts. As such, mice are the lowest sentinent species that can be used. The study of engineered sperm and cancer cells in culture (in vitro) provides us with clues on the mechanisms of the efficiency of sperm targeting and eradication of cancer cells in a simple and valuable context, which allows the establishment of hypotheses as to which spermbots will be the most suitable for the proposed purposes in a living animal. However, these systems do not recapitulate the complex cellular and organ interactions described above to allow us to investigate the impacts of sperm transport through the reproductive system on the targeting and eradication of early ovarian cancer lesions via the engineered spermbots that we will develop.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on published studies in this area, group sizes of between 5 and 8 animals (dependent on the readout, typically fewer for transplanted tumours compared to spontaneous tumours in genetically engineered mice) per experimental group suffice. We would typically examine more than one model cell subpopulation/line of ovarian cancer. Likewise, we may use several doses of a sperm(bot) load, several types of engineered spermbots, or several different drugs or treatment combinations together with the appropriate controls (unmodified sperm and components used to engineer the sperm individually) to test a hypothesis.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have taken advice from the local statistician within the Establishment and have adopted the principles described in the National Centre for the 3Rs' Experimental Design Assistant. As such, the use of mice will be minimised in several ways: (1) By considering on-going statistical estimation of power requirements in each of the studies, using prior results in order to use the minimum number of animals while retaining sufficient numbers for statistical significance. In general, we will use a sample size capable of detecting a 40% practical difference with 80% power and 95% confidence; (2) By incorporating as many test groups as possible within a single controlled experiment, reducing the number of controls required compared to a series of smaller experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies will be undertaken in order to determine the best approach and numbers of mice to be included in each treatment and control group in order to be both scientifically and statistically valid. The use of mice will be minimised in several ways: (1) By optimising our breeding programme to maximise use of mice in experimental protocols minimising the number of mice we do not require. Breeding is performed on a different Project Licence, by a dedicated group in our Establishment; (2) By doing as much preliminary work as possible in culture models in vitro and in situ prior to engaging in in vivo studies; (3) By minimising variability in results through utilising inbred strains and by housing them under identical conditions to limit variability; (4) By performing pilot studies using few mice when no information is available in the literature so that the number of mice utilised in experiments is reduced to minimal levels; (5) In all new protocols, we will establish the base line by procuring help and advice from husbandry staff and researchers at the Establishment but also from our experienced collaborators across the UK and internationally; (6) Furthermore, we will design small pilot experiments, carried out referring to https://www.nc3rs.org.uk/conducting-pilot-study, that will allow us to select the ideal conditions, so fewer animals are used, to calculate the minimum cohort size given the rate of expected events and also to determine gravity of these, allowing more accuracy for statistical powering calculation of group sizes in potential repeats.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The use of inbred and fully back-crossed mice not only reduces intragroup experimental variability but also eliminates incompatibility when cell transfers are carried out between various knockout, transgenic and wild-type strains. Our humane endpoints and check-points will help us keep pain, suffering distress and lasting harm to the mice to a minimum.

Why can't you use animals that are less sentient?

The cancer mouse models that we will use very closely recapitulate the human disease and thus will allow us to understand the molecular and cellular events and steps involved in the targeting and eradication of early ovarian cancer lesions developing inside the mouse oviduct by engineered spermbots. Since we are investigating ovarian cancer progression, we will need to use fully mature mice. As tumour growth is a long term process we cannot undertake the studies in terminally anaesthetised mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Where appropriate and possible, any short-term procedures will be undertaken under anaesthesia with the administration of analgaesia to minimise the experience of pain. We will constantly work to improve husbandry and procedures to minimise actual or potential pain, suffering, distress or lasting harm and/or improve animal welfare. Mice will be maintained in individually ventilated cages under barrier environment, to avoid infections. When considering which route of administration of substances to employ, we will use the least invasive route whilst maintaining direct control of dose.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Surgical procedures will be carried according to the Laboratory Animal Science Association Guiding Principles for Preparing for and Undertaking Aseptic Surgery. Unless otherwise specified, this project will follow the "Guidelines for the welfare and use of animals in cancer research" and the administration of substances and withdrawal of blood will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm (Morton et al., Lab Animals, 35(1): 1-41 (2001); Workman P, el al. British Journal of Cancer, 102:1555- 77 (2010)).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By reading 3Rs literature and participating in 3Rs workshops locally and nationally. Through discussing refinements with our Named Animal Care & Welfare Officer, Named

Veterinary Surgeon and Animals in Science Regulation Unit. I will be a regular attendee and contributor to our Retrospective Review and Licensees meetings and the 3Rs Poster session, all of which take place annually at our Establishment.

80. Implantation of Biologically Derived Tissue Scaffolds

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Tissue engineering, implant, stem cells, biomaterials, decellularization

Animal types	Life stages
Minipigs	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Scientists and clinicians have intended to replace a section of diseased organ/tissue with one which had been tissue engineered in the laboratory. This tissue engineered organ/tissue can restore normal function for the patient thereby improving their quality of life.

The aim of this study is to tissue engineer an oesophageal tract to replace and restore function of a section of oesophagus in minipigs.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

A diseased or dysfunction organ/tissue sometimes require a tissue repair or replacement to restore function. However, due to limited source of autologous or allogeneic tissue available, this treatment become difficult. The ability to use xenogenic sources, which will not be rejected by the host will play an important role in clinical application. The radical approach of decellularisation of donor tissues and repopulation of those tissues with host cells makes this type of transplantation or implantation possible.

What outputs do you think you will see at the end of this project?

The ability to supply organs or tissues for transplantation, possibly from xenogenic sources, which will not be rejected by the host, will transform the prospects of patients who currently suffer considerable morbidity due to failure of tissue function. The radical approach of decellularisation of donor tissues and repopulation of those tissues with host cells makes this type of transplantation or implantation possible. This will not only transform the lives and aspirations of the recipients but will also considerably reduce the financial burden to the NHS.

As we have developed 3D cell culture technology, we have had to develop more complex perfusion systems allowing for the assessment of cells and scaffolds simultaneously. The data generated is vital for scientific arena both in terms of the bioengineering concept but also in providing fundamental information on how cells interact with their environment and how their growth and development might be beneficially modified using growth factors.

Who or what will benefit from these outputs, and how?

The benefits from this tissue engineering technology is not restricted just to patients and health care providers, there are substantial benefits to the wider medical and pharmaceutical community. We have optimised a three-dimensional (3D) method to culture oesophageal, intestinal and hepatic cells as organoid units (OUs). The success of this project can also contribute to the tissue engineering and tissue regeneration scientific community.

How will you look to maximise the outputs of this work?

- The work will support researches to advance and improve scientific knowledge and to achieve scientific breakthrough in human health.

- Whenever possible, TGI adopt data management & sharing policy to maximise the outputs of researches through publication or other routes, thus to maximises benefits to health & society

- Whilst publication of results would not be discouraged, it is expected that in most cases publication or dissemination will not be possible as the research will be conducted as a commercial service to companies.

Species and numbers of animals expected to be used

- Minipigs: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Minipigs are selected for this study because of their size of oesophagus similar to human's. Minipigs grow slowly and steadily in size which will help the host oesophageal tissue to incorporate with the implanted tissue for the period of the study (6 months) in order to assess the function of the replaced tissue.

Typically, what will be done to an animal used in your project?

The animals will undergo some interventional procedures including surgery and general anaesthesia, some of the procedures are optional

- to remove or retrieve part of the tissues (up to four tissues) or organs (up to two organs)

- to implant biological derived tissue or tissue engineered scaffold onto the body, such as oesophageal section.

- to perform surgical intervention as part of the procedures for investigating some adverse affects

- to perform blood sampling to inject contrast media

- to perform non-invasive imaging such as CT scan or ultrasound

If repeat blood sampling is required for the experiment, up to two (usually one) indwelling catheters may be inserted and tunnelled through the skin. This would be done under general anaesthesia. Alternatively, if blood samples are only required occasionally, they may be taken directly from a blood vessel while the animal is restrained.

What are the expected impacts and/or adverse effects for the animals during your project?

Possible general adverse effects include reduce apatite, infection, tissue rejection, pain (moderate), bleeding, leakage and stricture in anastomosis and non function of implanted tissue. All these will be monitored and kept to a minimum.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

the overall expected severity level for all the animals included will be moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Tissue regeneration is a complex living body process. If we can show that the tissues/organs that we have prepared are appropriately functional in a complete physiological system (the whole animal) their use can be progressed very quickly into human clinical cases.

Which non-animal alternatives did you consider for use in this project?

Over the past we have been able to design many of our experiments so that more work was done in the lab before moving to the animals. By developing these 3D cell models we can take very tiny amounts of cells from any animal and grow the cells over longer periods and look to see how they react to different stimuli e.g. growth factors. We also hope to be able to offer these models to other scientists as well.

Why were they not suitable?

Even with every efforts to replace of using animal with other methods, some of the researches on biological response cannot be simulated or tested in the laboratory. Therefore, the animal is carefully selected to form an experimental model to achieve its research objectives. If we can show that the tissues/organs that we have prepared are appropriately functional in a complete physiological system (the whole animal), their use can be progressed very quickly into human clinical cases.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals that will be used under this licence before it expires are estimated through our past experiences and current demand from the study sponsors.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In order to reduce the number of animals being used in this project, a robust study design is essential to minimise the animal usage and to maximize the scientific achievement. Where possible, all tissues/organs for decellularisation will be retrieved from animals undergoing termination at the end of other projects thus reducing the need for specific donors. However, it may be necessary to use animals as specific donors if appropriate tissues/organs are not available. We will ensure that the number of specific donors is kept to absolute minimum.

- All initial research to establish proof of potential will be carried out using sophisticated bioreactors and 3D culture models thus eliminating the need for live implantation at these stages of the projects.

- As our in-vivo studies will aim to establish function of the recellularised tissues/organs, they are observational rather than statistically driven which means we will be able to use very few animals to prove function (typically 3-4 per tissue/organ) and progress to human clinical implantation.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All the studies will be carefully designed to use minimal number of animals without compromising the scientific output. This can be achieved from past study experience, published data, and pilot study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In order for some of the regenerated tissue/organ to be clinically relevant, the donor species need to be of similar size to humans. Therefore, the minipig is selected as the most suitable model for the duration of the study.

All the possible measurements will be carried out to minimise the pain, distress and harm to the animals, including staff training from external expert and closely work together with NVS and NACWA.

Why can’t you use animals that are less sentient?

Our choice of animal is in part dictated by human anatomical correlation. For this study, the minipig is a better anatomical model for oesophagus implantation. We, for both scientific and moral reasons, do not use dogs, cats or non-human primates.

As the most likely for future clinical application, xenogenic donor for human tissues/organs scaffold is from the pig, this will be the animal of choice for our tissue/organ retrieval and the technology

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All adverse effects will be closely monitored during post-op care period, which including pain management, which will be kept to absolute minimum.

Staff will be specially trained to handle minipig, and to observe any of these adverse effects. An video camera will be used to 24/7 monitor the minipigs

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

A number of published best practice guidance will be followed such as:

Guiding principles on good practice for Animal Welfare and Ethical Review Bodies,

ARRIVE: Animal Research Reporting In Vivo Experiments

Government Guidance on the Operation of the Animals (Scientific Procedures) Act 1986
LASA guidelines on surgery, anaesthesia and asepsis.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will closely work together with ASRU inspectors, our named information officer, NVS and AWERB; use of resources provided by the UK NC3Rs and Norway NORECOPA to update any advance policies, regulations and other information and impartment these accordingly and effectively during the project.

We will work closely under LASA guidelines on surgery, anaesthesia and asepsis, and seeking advice from specialist expert.

81. The Regulation of G-Protein-Coupled Receptor (Gpcr) Function in Health and Disease: Pharmacological and Genetic Manipulation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

GPCRs, Inflammation, Cardiovascular Disease, Type 2 diabetes

Animal types	Life stages
Mice	juvenile, adult, pregnant, embryo, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to elucidate how the function of certain membrane receptors called GPCRs impacts on whole animal physiology and how manipulation of these processes, either pharmacologically or genetically, influences disease progression and to identify new targets for drug discovery.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

GPCRs comprise the largest family of membrane receptors in the human genome, controlling almost all physiological processes, and are tightly regulated by a series of molecular events which serve to titrate signalling and prevent overstimulation. Disruption of these processes leads to disease. As such, GPCRs are highly sought-after drug targets by the pharmaceutical industry due to their 'druggable' nature i.e. being located on the plasma membrane and displaying tissue specificity. Indeed, therapeutics targeting GPCRs are available for almost all diseases including cardiovascular disease, mental health disorders, metabolic disease, cancer and inflammation. Despite their pre-eminence in the field, 80% of approved therapeutics target <20 specific GPCRs (out of ~350 tractable GPCRs).

However, it is currently unknown which receptors are involved in which physiological and disease processes and, in addition, many more have yet to be de-orphanised. These represent critical avenues for therapeutic intervention.

What outputs do you think you will see at the end of this project?

The projects and experiments performed will provide essential new information of GPCR contribution to health and disease and uncover novel therapeutics for pharmaceutical exploitation. This will be important for advancement of our understanding in the GPCR field as well as providing specific insight to GPCR dysregulation and disease. The data generated will subsequently lead to publications and presentation to the scientific community both national and international. There will also be significant public outreach to disseminate research findings to a lay audience.

Who or what will benefit from these outputs, and how?

The proposed research will identify novel GPCR and GPCR-effector targets that are important in the regulation of disease processes such as metabolic heath (obesity, type 2 diabetes and non-alcoholic liver disease or NAFLD), cardiovascular disease (atherosclerosis) and inflammation (sepsis).

Importantly, specific changes in GPCR molecular processes will be elucidated which, in the short term, will be a significant contribution to the field. In addition, longer-term, elucidation of whether pharmacological manipulation of GPCR function can reverse disease will have a profound impact on the pharmaceutical industry. The identification and development of new therapeutics with increased efficacy, reduced side effects and the

potential to reverse disease (as opposed to merely treat symptoms) will, overall, lead to improved patient health and a reduced cost and burden on the NHS.

How will you look to maximise the outputs of this work?

All work will be presented at national and international conferences and disseminated to the public during outreach and institute open days. In addition, all work will be published in peer reviewed journals. All work will be hypothesis driven and published regardless if confirmed or refuted.

Species and numbers of animals expected to be used

- Mice: 4000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice share ~85% homology of their protein coding regions with humans and are the lowest model that effectively recapitulates the human condition. Specifically, they express GPCRs highly homologous to human counterparts so that ligands are active at both species. This is not true of lower organisms (e.g. Flies and worms). In addition, disease models of metabolic health, cardiovascular disease and inflammation have been well established in mice to be directly relevant to the human condition. Finally, lower organisms do not have the complexity to adequately model higher complex behaviours or signalling networks.

Experiments performed in this license will use C57BL/6 mice or genetically altered (GA) mouse models on a C57BL/6 background. In addition, there are multiple genetically manipulated Cre lines commercially available that can be used in these studies. We will generally use mice from 8-10 weeks of age for our experiments and, for high fat/high cholesterol studies, animals will be kept on this diet for approximately 4-5 months.

Typically, what will be done to an animal used in your project?

We will use a wide range of techniques to address specific disease questions.

Metabolic health and cardiovascular disease. Mice fed a high fat/high cholesterol diet have increased weight gain, develop type 2 diabetes and, in genetically modified animals (ApoE-/-, LDLR-/-), have accelerated atherosclerotic plaque formation. We generally begin our experiments when mice are 8-10 weeks of age and are fed either chow or high fat/high cholesterol diet for a maximum of 20 weeks. During these experiments, mice be assessed for fat and lean mass changes using a Magnetic Resonance Scanner. Mice will be

individually placed in a tube and inserted inside the machine. The procedure will take ~5mins, after which the mouse will be returned to its home cage and will be performed multiple times (i.e. beginning, middle and end) per animal throughout the duration of the experiment. Mice will also be assessed for development of type 2 diabetes using glucose, insulin and pyruvate tolerance tests (GTTs, ITTs and PTTs respectively). Scanning and GTTS will occur 2-3 times per experiment and ITTs/PTTs once at the end. All tolerance tests will be performed a week apart in line with LASA guidelines and consist of a 5hr fast (overnight for PTTs) followed by blood glucose measurement. In addition, we will also assess whether pharmacological intervention (Intraperitoneally, I.P.) using drugs targeting GPCRs or GPCR adapter proteins will change these responses. Finally, we will use genetically altered transgenic lines to delete GPCR adaptor proteins exclusively in specific tissues to assess potential changesin disease progression.

Inflammation. To investigate the inflammatory response due to pathogen exposure, mice will be injected I.P. with the bacterial pathogen lipopolysaccharide (LPS) or peptidoglycan, or the fungal pathogen zymosan, that have been found to cause a mild inflammatory response and results in the recruitment and activation of neutrophils and macrophages. Typically for a C57Bl/6 mouse on chow diet, we would inject 1mg/kg of pathogen and cull 6h or 24 hrs post-injection and blood harvested for further analysis of immune cell composition. In addition, we will also assess whether pharmacological intervention using I.P. injection of compounds targeting GPCRs or GPCR adapter proteins will change these responses. Experiments may also be undertaken in transgenic mice exhibiting deletion of GPCR adaptor proteins exclusively in immune cells, leaving other tissues unchanged, to assess the impact of deletion in these cells on infection.

Terminal Culls. All mice shall be culled via Schedule 1 methods. For metabolic and cardiovascular experiments, mice will be fasted for 5h prior to culling and, in some cases, injected with insulin 10 mins before culling.

What are the expected impacts and/or adverse effects for the animals during your project?

Breeding

We do not expect any adverse effects during breeding of our genetically altered (GA) conditional and Cre lines.

Metabolic Measurements

No adverse reactions are expected during experimentation. For tolerance tests, mice will be injected

I.P. and blood collected from a single needle prick to the tail. This will result in mild discomfort that will be transient. Some drug interventions may result in weight loss during

HFD intake, however this should be beneficial to the animal. For experiments in modelling atherosclerosis, either the ApoE-/- or

LDLR-/- GA mice will be used. These will be obtained from commercial suppliers.

Inflammation

The dose of pathogen (LPS, peptidoglycan, zymosan) should only result in mild transient discomfort. Pharmacological intervention should result in beneficial to the animal and not cause additional stress.

Skin Conditions

In some cases mice have been found to develop ‘itchy ears’ and skin irritations due to scratching in response to HFD-feeding. These are usually treated with salt baths and topical medications. However, mice are culled if these persist.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect all mice to have severity level no higher than mild. The severity level for our GA mice in breeding is subthreshold. ApoE-/- and LDLR-/- mice used to model atherosclerosis have a moderate phenotype, but do not express any clinical symptoms when used under this project licence, therefore we expect mild severity and the same for our GA lines. For pathogen injection, all side effects are dose dependent and, for our scientific purpose we expect to be using doses that exhibit no more than mild severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice are the lowest model that effectively recapitulates the human condition. Lower organisms (e.g. Flies and worms) do not have the complexity to adequately model higher complex behaviours or signalling networks. By using mouse models we can investigate whole animal physiology that cannot be recapitulated in vitro and investigate genetic, protein and structural changes in tissues collected post-mortem as well as immune cell

analysis from blood. In addition, there are multiple genetically manipulated Cre lines commercially available that can be used in these studies.

Which non-animal alternatives did you consider for use in this project?

Prior to animal experimentation, all hypotheses are tested in vitro in cellular models to elucidate pharmacology before using animals as the final stage of investigation. Specifically, using immortalised cell lines expressing our proteins of interest, ligand affinity is determined using competition binding and efficacy via cell signalling assays. This ensures drug concentrations that fall within the ‘therapeutic window’ are selected before use in vivo and are not too low to be ineffective nor too high that would result in toxicity or off target effects.

Why were they not suitable?

In vitro systems do not recapitulate complex whole animal physiology, therefore animal experimentation is necessary.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

I have extensive experience and published data in the area of metabolic health, cardiovascular disease, inflammation and addiction. These have been used as resources to calculate the numbers needed for experimentation. For in vivo studies a sample size of 8-10 mice is required per group for most physiological studies. This may be increased to 12 per group if some tissues are required for both histological and RNA/protein analysis. For examples, hearts collected at terminal culls are too small to be analysed by more than one method, hence numbers will be increased to allow for analysis by several methods. Both male and female mice will be used as appropriate for the disease/experimental design.

Breeding will be required to develop GA animals from our conditional lines using tissue specific Cre transgenic mice. We will use the expertise from our breeding wing to ensure the appropriate number of breeding pairs are set up to allow lines to be generated with minimum wastage. Any surplus animals will either be culled, and tissues stored for future analysis, or offered to other users. We will also use our ‘non-crossed’ conditional mice as our wild type controls to ensure all mice are utilised.

Appropriate advice will be taken from suitably qualified statisticians within the institution in order that our studies are always undertaken using the minimal number of animals but retaining appropriate statistical rigour throughout.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All animal studies are preceded by extensive in vitro experimentation to elucidate the molecular pharmacology of the pathways being investigated and the NC3R's experimental design assistant consulted. We also have published extensively on the optimal number of animals required to perform each experiment. During terminal culls, most tissues are harvested along with blood, and stored until required, to ensure nothing is wasted and each animal used to its full potential. These tissues are also available to other collaborators within and out with our institute.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

At all points we will follow ARRIVE guidelines for all of our experimental set ups; blinding will be used whenever possible. However, for all the physiological testing, animals will be randomised in order to avoid bias. We will also consult the PREPARE guidelines to reduce waste and ensure reproducibility of experiments. Whenever possible, we aim to use both male and female mice to ensure relevance to the disease and explore potential sexual dimorphism in treatments.

Animal numbers bred for use on this Project will be minimised as far as possible by matching breeding to experimental requirements. Pilot studies, appropriate statistical analysis and power calculations will be employed to refine the number of animals used. The methods chosen will generate the greatest amount of data for the fewest animals used. To maximise the information gained from a single animal we aim to perform multiple in vitro analyses on each animal using the tissues obtained from terminal culls. Any pilot studies will be run in such a way that they will be rolled into the main study wherever possible, so that they are not additional to the numbers ultimately required for the main experiment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use C57BL/6 mice for all our experiments and all GA animals (ApoE-/-, LDLR-/-, tissue specific Cre, conditional knockouts) will be on a C57BL/6 background. This strain is used routinely for all experiments set out. For cardiovascular studies, ApoE-/- or LDLR-/- transgenics will be utilised since C57BL/6 mice do not develop atherosclerotic plaques. Furthermore, for LDLR-/- mice, require cholesterol in their diet to induce plaques, whereas ApoE-/- models spontaneously form these which can be accelerated via high fat/high cholesterol feeding.

For our metabolic measurements and experiments assessing atherosclerotic plaques formation, it will be required for mice to be fed a high fat/high cholesterol diet, to induce obesity and obesogenic pathologies. Type 2 diabetes development will be monitored via GTTs, ITTs and PTTs using the most refined ‘needle prick’ to the tail tip. In most experiments, only GTTs will be required in the middle and end of the study and in some cases, when comparing genotypes, at the beginning. PTTs or ITTS will be performed in the final two weeks of the study, a week apart. In some studies where obesogenic diet is used, pharmacological intervention could result in weight loss. However, this would be a beneficial consequence of the study and provide proof of principle that our intervention could be exploited for future therapeutic development.

We have opted to use I.P. injection for our bacterial inflammatory model, as less invasive than caecal ligation puncture.

Why can’t you use animals that are less sentient?

Less sentient animals do not have the physiology to accurately recapitulate the human condition.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In addition to keeping up to date with the most refined methods in the literature, the N3Rs database will be used as a resource for information regarding refinement. We will be kept up to date of any changes to procedures from our Named Information Officer and our Named Veterinary Surgeon. Additional training will be undertaken when required including reverification if needed. We will also consult with our experienced animal staff and Named Animal Welfare Officers to ensure the most refined practices are used at all times.

All procedures are the most refined and up to date methods to ensure minimal distress to the animal. Ear biopsies will be used to perform genotyping analysis as these give the least harm and stress to the mice. For tolerance tests, we will use a single needle prick to the tail rather than snipping the end that was used previously. We have also opted to use the AlphaTRAK 2 glucometers during these tests as found this equipment to be more sensitive and use less blood than other models. Echo MRI scanning used for body mass measurements do not require anaesthesia (unlike DXA analyses) and is undertaken in <5mins per mouse, ensuring minimal discomfort.

For the induction of bacterial infection, mice will be injected I.P. with LPS/peptidoglycan which is less invasive and more reproducible than caecal ligation puncture.

Mice will be handled routinely, prior and during experimentation, using the cupping and tubing method and group housed unless separated for welfare reasons (i.e. fighting).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidance for the project will be taken from resources provided online by NC3Rs, ARRIVE, Norecopa, PREPARE (hubs and microsites) and LASA guidelines for administration of substances. This includes links to publications, other online resources, and video and training materials. The vast array of resources guide on the general principles underlying the experiments highlighted in this project, including anaesthesia, breeding strategy and numbers, and experimental design. Changes in current guidelines will be implemented to the project in line with current literature and published reports. We consult with experts to implement improvements in animal welfare. Local refinements will also be implemented.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly receive updates in advancement from the N3Rs newsletter. In addition, our Named Information Officer is involved with several databases and forums to keep informed of any changes to procedure.

82. Studying the Regulation of Immune Responses in Cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Cancer, Immune response, Immunotherapy, Vaccines

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of the project is to understand the processes that support the initiation of immune responses to cancer to enable the identification and design of immunotherapeutic approaches for clinical benefit. The specific aims are:

1. Determine the mechanism of action by which immune responses to cancer are regulated (both positive and negative), and how tumours are able to influence this regulation.

2. To test the hypothesis that manipulation of these mechanisms can enhance immunity to tumours and be exploited in vaccines.

3. To evaluate potential new therapeutic agents targeting these pathways of regulation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

ne in two people in the UK will be diagnosed with cancer in their lifetime with 375,000 new cases of cancer in the UK every year and >165,000 deaths. There have been significant advances in screening, diagnosis, and treatment of cancer, however, fewer than 50% of people will survive the disease for greater than 10 years. Current treatments of cancer, which have been developed over the last 40 years, typically involve surgery together with chemotherapy and/or radiotherapy and are effective in some cancers improving patient survival. Despite this, one of the main issues with these therapies is that for a significant proportion of patients these treatments fail, and the cancer will return. Over the past 20 years, it has become evident that the immune system is important in detecting and combatting cancer. Crucially, recent developments in therapies that harness the immune system (immunotherapy) have shown significant advances in improving patient outcomes in some cancers. However, these immune responses are variable and only observed in a small minority of patients following treatment.

Therefore, having a better understanding of how cancers interact and regulate immune responses will help to enable the development of new more effective cancer therapies to improve patient outcomes.

What outputs do you think you will see at the end of this project?

Immunotherapy allows us to treat patients with therapies specific to the tumour, either utilising or ‘educating’ the patient’s own immune system to destroy cancer cells while leaving normal tissue mostly untouched. It is envisaged that this approach would mean that unlike conventional treatment, antibody treatment and vaccination (including changing how tumours look to the immune system) should not be associated with long lasting harm to the individual. Vaccination would lead to the development of a memory of the cancer cells meaning that the immune system would continue to search for, recognise and kill cancer cells long after the treatment has ceased providing long-term protection. Despite this promise, current immunotherapies work in only a minority of patients with variable responses in different cancers. In this project we therefore aim to understand how immune responses to cancers are regulated and how they are manipulated by cancers. Through this information we hope to gain better understanding of why some treatments fail. This

would allow us and others to develop new therapies to overcome these barriers and improve patient survival.

The overall aim is to gain a greater understanding of the way in which cancers are detected and identified by the immune response. If successful, this will allow the identification and design of therapies that harness the immune system for patients to better target their cancers. In addition, an understanding of the immune processes that support immune responses that target and kill cancer will permit continued monitoring of anti-cancer responses during remission and allow very early signs of relapse to be investigated. The ability to predict response to immunotherapy is also vital as current treatments works in only ~5-25% of patients. During this programme of work we anticipate the development of new drugs and approaches and will collaborate with partners such as charities and biotech/pharmaceutical companies to bring these to clinic. A drug that was developed under the current licence is scheduled for first in human trials.

Our findings will be made available to other scientists through publication in peer-reviewed journals and presentations at scientific conferences and meetings. Under the current project licence, we have published 14 papers in well regarded scientific journals and have presented our findings at 12 international scientific meetings and participated in public outreach activities and events. Together, the outputs of this work will advance fundamental scientific knowledge of how cancers are detected and interact with the immune system and the induction of protective immune responses. The work in this project will contribute to the early detection, potential new treatments and management of cancer for both clinical and veterinary applications.

Who or what will benefit from these outputs, and how?

Our studies will have multiple beneficiaries. In the short term we will publish our discoveries in open- access peer-reviewed journals, present at scientific and clinical conferences and participate in public outreach activities. These manuscripts will be available on eprints (online and freely accessible university repository) 3 months after publication. This data will be of particular interest to other scientists, clinicians and biotech/pharmaceutical companies. This new knowledge will provide others with a greater understanding of immune responses to cancer and enable development of more effective therapies in the future. The development of new therapies that benefit cancer patients is a long-term impact. In addition to cancer, the knowledge of how immune responses are regulated by cancer will be of interest to other research fields. There are direct parallels between cancer and infectious disease in the need to induce strong immune responses to clear either the cancer or the infection. Therefore, new knowledge from the programme will be of benefit to both human and veterinary clinical applications in this area. Another application could be in autoimmunity where the patient’s immune response attacks their own body. By understanding how immune responses are regulated, we may also gain a greater understanding of how to turn off overactive immune responses. This could be used to develop new therapies.

How will you look to maximise the outputs of this work?

We will aim to maximise our outputs through publication and presentation of our findings. We will publish our findings in open access journals and ensure all raw data is disclosed. This will enable them to be accessible to all. In addition, we will publish early drafts of our findings on bioRxiv which is a pre- published archive for life sciences and copies of our publications are available on the University ePrints server. This will make our findings immediately available to the scientific community. We have a strong track record in reporting our findings with 14 papers published during the current licence and include approaches that were ultimately unsuccessful to prevent unnecessary replication of this work. Most of the work undertaken is collaborative, involving other research groups and/or pharmaceutical companies. This collaborative approach further ensures that we can maximise our outputs.

Species and numbers of animals expected to be used

- Mice: 2900

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We make every attempt to reduce the use of animals by using in vitro methods, for example isolating and performing experiments on various blood cells that are an essential part of the immune system outside of the body and creating new reagents from these cells for future use in vitro. However, as we are examining interactions between various arms of the immune system in different tissue and organs, in vivo investigations must be undertaken. The animal models we use are essential in providing the proof of concept and efficacy in an amenable system that can be manipulated to define the underlying mechanisms of action to translate successfully to humans; something that is not possible in humans.

Adult mice will be used in this study as they have a mature immune system, which is well characterised, with the cellular and molecular interactions broadly similar between mice and humans. This allows investigations of immune mechanisms and immunotherapies in these animals that are likely to translate to humans. The use of inbred mouse strains ensures that there is reduced variability in our experiments enabling valid conclusions to be drawn from the data obtained while minimising numbers of animals used. Our use of a number of tumour models that have been established in mice, whilst not fully replicating human disease, allow specific questions in relation to anti-tumour immune responses to be addressed to enhance the potential for translation into humans.

Typically, what will be done to an animal used in your project?

This work will involve a series of experiments to examine immune responses to cancer. This will be performed by introducing immune-modulatory agents and assessing their effects on the immune system and tumour cells.

In experiments to isolate specific immune cells, which can be maintained in the lab, mice will be injected with material to stimulate an immune response over a period of weeks (typically three weeks) before the mice are killed and their immune cells isolated to use in the lab.

In experiments to assess immune responses to vaccines or immune stimulatory agents, mice will be injected with a vaccine or stimulatory agent typically on three occasions (at least 2 weeks apart). The immune response is measured by taking peripheral blood to assess changes in immune cell populations over a period of weeks. A proportion of mice (85-90%) will be killed typically after 3-4 weeks, and their tissues examined to understand how the immune response is developing in different organs. These experiments are often done in the presence of a growing tumour, injected earlier in the experiment. This allows assessment of anti-tumour responses and to understand if immune cells are homing to the tumour. A proportion of mice are followed to measure tumour growth over time in experiments that may last up to 60 days.

We constantly endeavour to use the most appropriate model to address our objectives and therefore periodically we acquire or develop new tumour models which need to have their humane endpoints established to ensure we can reproducibly use these models to achieve our objectives whilst minimising the impact on welfare. Typically, 3-5 mice will be injected with tumour cells by an appropriate route and tumour growth and animal welfare monitored to determine the humane endpoint and to ensure no unexpected or excessive harms are evident.

What are the expected impacts and/or adverse effects for the animals during your project?

We typically identify mice by ear notching – this is expected to result in only mild and transient pain with no healing problems.

Mice will be injected with various substances that can stimulate immune responses using a combination of different volumes, routes, and frequencies. Injections will cause momentary pain which will be minimised by administering analgesic to the area and using the smallest needle size.

Substances may also be given orally to ensure they are taken up effectively. This can cause mild and transient pain and discomfort, but this resolves quickly. The minimum number of administrations and routes will be used to achieve the scientific objectives. Typically, animals will receive no more than three intraperitoneal injections, or five oral

gavage doses a week for 1-2 weeks. On some occasions mice may receive two injections on a given day through a combination of different routes.

For some experiments, mice will receive tumour cells by subcutaneous (under the skin) injection. The tumours grow at this site and can be readily monitored and measured over time using digital callipers. Mice are euthanised prior to or when the tumour reaches a size that is deemed to impact their normal behaviour, which may vary for different tumours, based on previous research. Throughout, the health status of the animal is the primary consideration. Experiments will be terminated, or individual mice euthanised at the earliest signs of tumour-associated symptoms such as piloerection, restricted movement, abnormal posture, abnormal gait, hunching, and/or weight loss up to, but not reaching, 20% body weight.

In some experiments which measure immune changes, peripheral blood is taken from a superficial vessel. Pain from bleeding is controlled by suitable anaesthesia/analgesic and no long-lasting harm with minimal volumes being taken.

When the immune system is stimulated, for example with immune stimulatory substances e.g. vaccines, this can result in symptoms similar to those experienced during an infection (lethargy, fever etc.). This can result in mice becoming less mobile, exhibiting pilo-erection etc. Typically, these symptoms are transient (first few hours) but can recur as the immune response develops (e.g. after several days) potentially resulting in further effects such as weight loss. These effects are therefore carefully monitored with mice killed if their symptoms become more severe or pass defined humane endpoint

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

During our current project licence, the proportion of mice experiencing sub-threshold, mild, moderate or severe severities was as follows and we expect similar proportions in our new project licence:

Sub-threshold – 2.5%

Mild – 71.2%

Moderate – 26.3%

Severe – <0.1%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Every attempt is made to reduce the use of animals by using in vitro methodology (in vitro experiments on mouse and human T cells and tumours, generation of T cell hybridomas). The generation of T cell hybridomas serves as a direct in vitro replacement for the need to generate T cell reagents from the immunisation of mice. This ensures that we can remove the requirement for multiple mice and reduces the variability of responses observed. However, as we are examining the immune system and the interactions between different tissue and organs, in vivo investigations must be undertaken. The animal models we use are essential in providing the proof of concept and efficacy in an amenable system that can be manipulated; something that is not possible in humans. Studying the complex interactions between various arms of the immune response in different tissues and organs is currently not possible using in vitro systems, therefore systemic in vivo studies are still necessary to study these complex immune processes.

Adult mice will be used in this study as they have a mature immune system, which is well characterised, with the cellular and molecular interactions broadly similar between mice and humans. This allows investigations of immune mechanisms and immunotherapies in these animals that are likely to translate to humans. The use of inbred mouse strains ensures that there is reduced variability in our experiments enabling valid conclusions to be drawn from the data obtained. Our use of a number of tumour models that have been established in mice, whilst not fully replicating human disease, allow specific questions in relation to anti-tumour immune responses to be addressed to enhance the potential for translation into humans.

Which non-animal alternatives did you consider for use in this project?

During the current licence we have been able forge links with clinicians which has enabled us to gain access to primary human material (tumours, blood, lymph nodes) to study human cancer more directly. These tissues allow us to reduce our requirement for animals, however these models are ultimately limited compared to the complexity of studying holistic in vivo responses and whole body systems. We therefore still need to use mice to study the influence of tumours on immune responses and how they are regulated using appropriate mouse models.

In a further attempt to replace the use of mice, we have generated a library of research tools such as T cell lines/clones and hybridomas that can be used in in vitro assays as a replacement for freshly isolated T cells from immunised mice. We have also explored the use of in silico methods to aid the prediction of immune responses. In some instances, this

allows us to predict the expected immune responses following vaccination and tumour challenge replacing the need for in vivo experiments.

It is difficult to quantify the reduction in the number of mice these replacement strategies have resulted in as the experiments are not directly interchangeable and form the basis for answering different scientific questions. However, the greater use of human material and the generation of research tools that can be used in vitro, enables us to replace the need for mice in our studies.

Why were they not suitable?

We are investigating the therapeutic effects of vaccines and examining the interactions of various arms of the murine immune system, with a view to therapeutic applications in humans. These interactions occur between multiple cell types, different tissues and organs, and as such they cannot yet be effectively reproduced in vitro. Whilst we can model particular aspects of the immune response in different in vitro systems, these currently do not fully recapitulate all facets of an intact immune system in a whole organism and therefore in vivo models are fundamental to our studies. In addition, they do not allow the assessment of unknown interactions throughout the body, which are critical to observe in developing new therapeutics e.g. liver, kidney toxicity.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimate of the expected number of animals is based on the annual usage of animals in the current project licence whilst accounting for reductions in number for this new project licence compared to the current licence. This new project licence represents a reduction of almost 70% in comparison to our estimate from the current project licence. This reflects our shift to use primary human tissues where possible and strategies employed to replace the need for animal experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our experimental design is always based on the consideration of how we can achieve meaningful and reproducible results whilst using the minimum number of mice. We are committed to reducing animal waste, implementing the three Rs, and maximising the

reproducibility of research and so follow the PREPARE guidelines (https://norecopa.no/prepare) when considering our experiments.

As a means to reduce the numbers of mice in our project, inbred strains of mice will be used to reduce variability and allow reduced numbers whilst delivering reproducible data. In addition, we routinely use age- and sex-matched mice that have been bred in the same facility to further minimise variability.

Furthermore, developments in techniques and assay sensitivity (e.g. tetramer staining, ELISpot, intracellular cytokine staining) permit the detection of very specific immune responses in individual animals, rather than pooling animals from a larger group for analysis, allowing a reduction in the total numbers of animals required.

We have been using a range of tumour models for some time and so are aware of the reproducibility of controls and appropriate mouse numbers required in tumour growth and therapy experiments. Where we are studying new models or treatments, we will first confirm expected humane endpoints and then perform pilot experiments to gain more understanding and knowledge for the design of larger studies. This includes aspects such as dosing regimens, as well as to monitor for any signs of adverse events. Typically, these early experiments will consist of small groups (two mice) inoculated with a new tumour at different cell numbers to establish the tumour growth kinetics etc., even where data exists in the literature, as we appreciate the impacts of the local environment, microbiome etc on immune responses and tumour growth kinetics etc.. We also seek to minimise experimental bias through the use of empirical measurements where appropriate in relation to humane endpoints, such as the use of callipers to measure tumour size in tumour challenge experiments.

Where we have useful data (typically from pilot experiments), power analyses will be used to help guide the optimal numbers of mice needed for each experiment, taking into account expected magnitudes of impact. Power analysis is performed using the PS: Power and Sample Size Calculation programme: www.biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize):

For example, when assessing the immune responses in mice to immunotherapy experiments in either of the two commonly used in-bred strains of mice (C57BL/6 and Balb/c), we estimate that the difference in average time to reach humane endpoint that we need to be able to detect a significant effect due to the immunotherapy is 10 days e.g. tumour inoculated mice treated with immunotherapy survive 10 days longer than the vehicle treated tumour inoculated control mice the typical time to reach humane endpoint for control mice is 25 days. Using this information, a power calculation based on the ability to identify a difference between the groups indicates that we need a total of six mice per group. From our experience, we have found that we can reduce this number to five mice per group to enable us to detect a difference in immune response between treatment and control groups in the tumour models we use (CT26, B16F10, MC38). Similarly, for

assessing immune responses to vaccines or other immunostimulatory agents we typically use five mice per group for each experiment as this allows us to identify relatively small difference between groups (10-20% reduction in expression of Major histocompatibility complex molecules on the surface of immune cells) at the 5% significance level.

Therefore, the proposed mechanistic studies will be performed with experiments using groups of 5 mice in a standard vehicle versus drug manner. Throughout, replicate experiments will be performed to ensure reproducibility and when appropriate such studies may be combined to increase sample size (e.g. therapeutic survival). To assess animal survival Kaplan Meier curves will be analysed by Log rank test.

If substances or tumour models are used for the first time, pilot studies will be performed on individual mice and humane endpoints established as indicated above. Typically, substances will be administered and monitored within the first hour and then after 4 and 24h for adverse effects before proceeding; a dose escalation schedule conforming to accepted practice will be used should adverse effects be predicted from literature or expected mechanism of action. When agents are used for the first time in the laboratory, their dose will be based on previous published data whenever possible.

When new tumour lines are introduced, they must go through quarantine to ensure no animal pathogens are transferred to the facility and ensure their safe introduction into the animal facility. As an additional measure of reduction, we use this period to also monitor growth and humane endpoints reducing the need for additional animal use.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Mice used across experiments are inbred thereby minimising intra-group variability and allowing reduced mouse numbers for experiments. Experiments are always designed with the least number of animals that allow us to obtain statistically valid results. We use Power analysis calculations to determine the numbers of mice required to deliver statistically significant results, although with established disease models with which we have experience we can often use smaller numbers of animals whilst retaining our ability to achieve statistical significance.

Developments in technologies, techniques and assay sensitivity have enabled detection of very specific immune responses and hence more information to be obtained from one individual mouse than was previously possible e.g. multi-parameter flow cytometry, thus enabling multiple parameters to be assessed simultaneously from small samples. These higher dimensional analysis tools reduce the need to kill multiple mice at different time points. As an example, we now routinely use multi-parameter flow cytometry that allows us to look at 16 pieces of information in individual cells from mice where previously we were only able to assess 3-8. Soon we will be expanding this technology to allow us to look at up to 40 pieces of information further reducing the amount of material needed.

Other optimisations to reduce animal use include:

We routinely freeze additional animal tissues (whole and as single cell suspensions) when a mouse is killed to provide controls in in vitro experiments reducing the need to kill mice specifically for this comparative purpose.

The in vitro use of immune cells isolated from mouse tissues offers several benefits and aids in optimising the number of animals used for in vivo experiments. Importantly, this strategy is in line with the 3Rs. Due to the relatively low numbers of cells required to perform in vitro assays, this experimental approach allows the screening of immune regulating drugs or combinations, with potential for in vivo activity, whilst minimising the number of animals required. Furthermore, they allow refining doses, and identifying potential mechanisms of action and adverse reactions, prior to in vivo testing. In addition, in vitro assays using mouse immune cells represent a powerful reductionist approach to study their activation. They also allow the isolation and analysis of distinct cell subsets, and the direct assessment of drugs on each individual cell population, otherwise difficult to accomplish.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Adult mice will be used in this study as they have a mature immune system, which is well characterised, with the cellular and molecular interactions broadly similar between mice and humans. This allows investigations of immune mechanisms and immunotherapies in these animals that are likely to translate to humans. The use of inbred mouse strains ensures that there is reduced variability in our experiments enabling valid conclusions to be drawn from the data obtained. Our use of several tumour models that have been established in mice, whilst not fully replicating human disease, allow specific questions in relation to anti-tumour immune responses to be addressed to enhance the potential for translation into humans.

Our investigations of how immune responses are regulated or altered by tumours typically involve the use of in vitro approaches to first establish that we can observe differences that we expect. These experiments may include the ability of immune cells to kill tumour following treatment with a reagent. If successful we would progress to pre-clinical animal tumour models. This is a necessary step as the use of in vitro cell lines are not able to fully

recapitulate the complex interactions between multiple different cells and tissues and hence the need for animal experiments. In experiments where we are assessing new reagents that alter the immune response, we would first examine their effects in the absence of tumour to assess ensure any changes are observed without unnecessarily increasing the welfare impact to the animals by adding the cumulative harm from additional interventions and/or activities of the tumour. In all experiments we ensure humane endpoints are established that minimise harm to the animal without compromising the validity of the data obtained.

Why can't you use animals that are less sentient?

Our work examines the regulation of the immune system which is a complex system involving many different elements throughout the body and organs that work together to target foreign pathogens or cancer. The cellular and molecular interactions of the mouse immune system are broadly similar to that of humans, and therefore allow the investigation of potentially clinically relevant immune altering and/or activating treatments and their mechanisms of action in these animals. The use of less sentient species is not appropriate as they have very different immune systems as key components are either absent or are not as complex e.g. nematodes do not possess adaptive immunity. Younger animals also do not display the mature, antigen experienced, immune systems (the immune system becomes educated with age and development) that we seek to investigate and is most relevant for translation to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We continually review our procedures and protocols and if unnecessary harms are identified or can be reduced, we modify protocols through discussion with the NACWOs, PPL holder, academic lead for the animal facility and Named Veterinary Surgeon (NVS) as required.

Over the period of the current project licence, we have instigated a number of changes that are listed below that serve as examples of our commitment to refinement:

1) We have moved to a new purpose-built animal facility. This unit is more conducive to high quality in vivo research utilising a 'barrier' system meaning that all materials coming into the unit are clean and enter via a dedicated positive-pressure pass-through hatch. All personnel entering must now change into scrubs and pass through an air-shower on entering (and leaving). The new facility also houses a separate quarantine facility that is no longer inside the main unit, but attached and accessible separately, with dedicated technicians only permitted.

2) All new strains of mice entering quarantine undergo rederivation before offspring are allowed in the main unit. All new cell lines must be screened in mice in the quarantine room, and serum from these mice and sentinels are assessed for the presence of

pathogens before cells are allowed to enter the main unit. All mice are now housed in individually ventilated cages. These changes minimise the risk of infection with obvious potential implications for the welfare of the animals and the quality of the research. In addition, the IVCs permit less disturbance to the animals as an additional refinement.

3) We have also implemented a number of new policies to improve welfare and reduce adverse experiences. All mice undergoing an injection now undergo a 'second check' by the person performing that procedure, within 30 mins-4 hours. This was instigated to prevent animal welfare impact should an unanticipated adverse event occur. If a procedure is delegated to a technician, this second check also incorporates a 'positive handover' between the technician and the PILh to transfer the responsibility for the mice back to the PILh to ensure it is always clear where responsibility for welfare ultimately lies.

4) In response to changing guidelines several further and specific refinements have been put in place. These include:

(a) Mice are handled by 'cupping' or by gently moving them using a dedicated tunnel, prior to any restraint to ensure mice are calm and habituated to the person performing injections, serving to reduce anxiety when handling mice.

(b) A policy has been instigated that needles are only to be used once. This single use policy is to prevent mice receiving an injection with a blunted needle. (c) We have transitioned towards the use of venesection as the primary method of taking blood samples instead of tail tipping. While this is not always possible (for instance when mice are receiving concurrent intravenous injections) new PILh are now trained in venesection and established PILh who regularly bleed mice have undergone re-training.

(d) We have instigated a policy that new in vivo experiments must be detailed on a 'study-plan' that encompass the experimental aims and how they address the project licence objectives, number and strain of mice, substances/cells administered, treatment dates, confirmation of required competencies and/or delegation of procedures, any known or anticipated adverse effects, welfare measures and monitoring employed and appropriate risk assessments. This study-plan must be submitted to the project licence holder and then responsible NACWO for approvals prior to experiment commencement. This has ensured that there is evidence of an appropriately detailed dialogue between the personal and project licence holder prior to work and as necessary enabled a discussion about the number of mice used and procedures administered.

5) We also have a new system that records training competencies for individual PILh to ensure that training on all procedures is renewed every 3 years. Training is conducted by dedicated trainers using Directly observed practical skills (DOP) forms written specifically for this purpose. As the project licence holder, I have access to these records, and reference is made to them on the 'study plan' ensuring that PILh also review their records regularly.

6) We have also adopted the practice of transferring male mouse nesting material, not substrate, to minimise male mouse aggression (the nest has calming pheromones in it, but the sawdust aggravates aggression as it holds the testosterone).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are a number of detailed publications and guidelines for the welfare and use of animals in cancer research that provide excellent guidance in the methodologies, study design and best practice that we will follow and adapt as appropriate to our research (Guidelines for the welfare and use of animals in cancer research Workman, P., Aboagye, E., Balkwill, F. et al. Guidelines for the welfare and use of animals in cancer research. Br J Cancer 102, 1555–1577 (2010). https://doi.org/10.1038/sj.bjc.6605642).

We will follow the ARRIVE guidelines https://arriveguidelines.org/ which provide a checklist of the minimum information required to be reported by groups using animals in research. ARRIVE guidelines are essential to help overcome issues in science such as reproducibility, reducing bias and the correct use of statistical methods of analysis.

In addition, we will follow and consult NORECOPA https://norecopa.no/3r-guide : Norway's National Consensus Platform for the advancement of the 3Rs (Replacement, Reduction and Refinement associated with animal experiments) database platform and PREPARE https://norecopa.no/prepare (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for better science experiments using animals to ensure that we are using the best models for our research.

Finally, regular communication with other users of tumour models at the University and communication with our peers at seminars and conferences and in published literature ensures any new or updated best practice is identified and adopted. Best practice information is also disseminated by our Named Information Officer.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have a very active users' group that meet regularly (~3 times a year) to discuss issues that arise within the animal facility. These are attended by personal licence holders (PILh), project licence holders (PPLh), NACWO, Home Office Liaison Contact (HOLC) and establishment licence holder. Any issues and incidents that PILh need to be aware of are discussed at this meeting. In addition, the BRF user's meeting are used to disseminate information from Animals in Science Regulation Unit (ASRU), National Centre for the replacement, Refinement and Reduction of Animals in Research (NC3Rs) and other organisations and to provide details of training opportunities to enhance welfare and research practice. We have also begun a strategy of ensuring that PPLh and PILh undergo

regular refresher training (at least every 3 years) to ensure that they remain up to date with changes to best practice and aware of their responsibilities under the Animals (Scientific Procedures) Act 1986 (ASPA). These advances are further communicated through our active team of NACWOs and regular email updates.

We will also stay up to date with specific cancer groups, databases and alternatives (NC3Rs) for cancer models such as https://resources.researchanimaltraining.com/faqs/breast-cancer-research- alternatives-database; https://data.jrc.ec.europa.eu/dataset/352f7dfd-05cf-434b-a96a-7e270dc76573

83. Understanding the Balance of Immunity in the Skin in Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Skin, Immunology, Cancer, Dendritic cells, Immunotherapy

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this work is to use our study of skin disease to better understand how immune responses are controlled in healthy and diseased skin, and how cancer might exploit skin immune responses in order to grow and spread.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The skin is an important barrier site, but it is also the most common site of disease. In particular, the skin is prone to diseases caused by the inappropriate activation of white blood cells called T cells, either due to auto-immune disease or when patients take drugs that activate their immune system. Most people are also unaware that skin cancers are more common in the skin than any other site.

Because most skin diseases are not life-threatening, research into skin disease has lagged behind other diseases, but these diseases seriously impact on quality of life due to discomfort and disfigurement. We need to understand how immune responses are balanced in the skin in order to work out why they go wrong so easily and how we can being to prevent it from happening. In particular, we are focused on specialised groups of innate immune cells called dendritic cells and Langerhans cells. We know that these cells are important for detecting foreign invaders in the skin, but also for making sure that we don't start immune responses to harmless insults. But, we don't understand what the signals are that allow these cells to live in the skin and how these changes when things go wrong in disease.

Cancer is caused by our own cells that become changed so they can grow without control. Our immune system can be taught that cancer cells are a problem and need to be destroyed. This approach is called cancer immunotherapy, and has led to a dramatic change in our treatment of cancer in the last decade. But these treatments still don't work in most patients and we need to know more about how immune cells interact in tumours in order to find new ways of improving outcomes. One of the problems is that T cells activated by treatments to kill tumour cells can attack healthy tissues to cause graft-versus-host disease (GVHD), and the skin is one of the worst affected sites. This can lead to complete breakdown of the surface of the skin and patients die from dehydration and infection. This means that GVHD is a major problem limiting the success of these cancer treatments.

What outputs do you think you will see at the end of this project?

This project will provide new information about the way immune cells talk to each other in the skin. From this, we can begin to learn why this goes wrong in skin diseases like graft-versus-host disease (GVHD) in people who have received bone marrow transplants, and also other so-called autoimmune disease where the immune system attacks the skin. This work will be published in journals that can be read by people around the world, and we will also talk about our findings with other scientists at conferences in the UK and abroad.

Our work investigating dendritic cells in tumours will also provide new information that we will publish and share. We hope that this work could lead to new ideas about how to improve cancer treatments that work by activating our immune systems to destroy cancerous cells.

Who or what will benefit from these outputs, and how?

In the short-term (3-5 years) the main beneficiaries from this work will be members of the academic and biotech research communities. Understanding how immunity is regulated at barrier sites such as the skin is becoming increasingly important as we live in an aging society, where more and more people are suffering from diseases caused by activation of immune cells. We hope that in the long- term (10 years+), our studies will lead to better ways of treating people with skin diseases.Treatments for cancer have also improved significantly in the last decade, but there are still too many people who do not respond to treatment, and some of the new immunotherapies can cause side-effects in the skin. We still don't know what the long-term effects of these treatments will be on individuals.

How will you look to maximise the outputs of this work?

We aim to publish all novel, robust research findings, whether results represent new findings and insights or simply support a null hypothesis. Storing manuscripts with pre-print servers will help to maximise speed of spreading new data and prevent other labs duplicating work un-necessarily.

As part of our work we also generate large datasets, for example of gene expression, which we will share by depositing it on publicly-available databases. The advantage of these datasets is that they become a resource for the lab, to be mined over time as projects mature and we develop new hypotheses.

Species and numbers of animals expected to be used

- Mice: 15500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The aim of our research is to better understand how the immune system is maintained and functions in healthy skin and disease. Because of the complex physical arrangement of the immune system, and migration of immune cells between different sites, we need to use models that have the blood flow and other tissue features that allow cells of interest to meet and interact. We still cannot fully replicate this anatomical detail using in vitro models. The mouse is the best system to study the immune system; we know that the types of immune cells and the way they work are very similar between mice and people, and the immune system of the mouse, next to that of human, is the best characterised immune system of any organism. This means there are lots of tools and reagents available to study our research questions. We can also genetically manipulate mice, which is a key tool to unravelling the importance of important factors that allow immune cells to

communicate and do their job. We are focused on using adult mice for our work because we want to understand how immune cell changes to the skin in adults can be repaired.

Typically, what will be done to an animal used in your project?

All our mice are kept in sealed "individually ventilated cages" in groups of up to 5 mice. This means that they have a clean air supply and they are also given clean bedding and food and other items such as cardboard tubes to make the environment as comfortable as possible for them. We need to keep the mice in these special cages because sometimes the experiments mean that their immune systems are not working properly, so we have to protect them from catching diseases.

Healthy mice will typically receive an intervention to perturb skin immune cells, for example we might paint something onto the skin that we know will activate Langerhans cells. We normally use the back of the ears for these experiments because there is less fur there and it is a fixed area of skin. Mice might also received injections of drugs or antibodies to get rid of different immune cells to see how the response to what we have painted on the skin changes when certain immune cells are missing. When we give drugs this might be by different routes, but usually we try to make sure the drugs reach the skin immune cells so we inject them sub-cutaneously or into the dermis (intra-dermal). Sub-cutaneous injections are very quick, we pick up the mouse and hold it in a secure way so it does not move, then inject the substance under the skin usually at the side of the body or between the shoulders. The experience for the mouse will be very similar to when we have vaccinations in the arm. Intra-dermal injections will be more painful so we use a gas anaesthetic to put the mouse to sleep before injecting the drug into the skin on the side of the body. As soon as the mouse is moved away from the nozzle with the gas they wake up and are running around the cage within less than a minute. Sometimes we need to inject drugs or antibodies directly into the bloodstream of the mouse to make sure that they have an effect all over. We also take blood samples from some mice when we want to track changes in immune cells over time. Here injection is into the tail vein because it is the easiest vein to access with the least discomfort to the mouse. We need to warm the mice up so that we can see the vein so we put them in a special dark box with a carefully controlled heating system than heats to no more than body temperature for a maximum of 10 minutes. The mice are then placed in a tube-shaped restrainer that is the right size to keep them still while their tail is exposed, while also being large enough not to squash the mouse or restrict breathing. The person doing the injections will receive training for tail vein injections and usually (90% of the mice) the injection itself is very quick with only very short-term discomfort to the mouse. However, for some of our experiments we have to use older male mice and here the skin on the tail can be thicker, making it harder to see the veins. To minimise the suffering for the mice we start our injections about a third of the way down the tail from base, and only try 3 times at sites moving towards the tail base. If this does not work then the mouse is returned to the cage without injection. A similar procedure is used to sample the blood except a needle is use to puncture the tail vein with a small hole and special tubes used to collect the drops of blood that form. We only collect

very small volumes of blood (typically 50-100 microlitres) which is less than 10% or the total blood volume. When we have enough blood, pressure is put on the puncture site to stop bleeding and the mouse returned to the cage. In all out experiments with healthy mice, these interventions are quick and

the mice rapidly recover when returned to their cages. The substances we inject have all been used by our lab in the past and by other researchers and are given in amounts that we know do not cause discomfort or suffering to the mice. We then check the mice regularly before they are killed by a humane method (usually between 1 to 4 weeks from the start of the experiment) and tissues taken for analysis after death.

An important part of our work is investigating how Langerhans cells and dendritic cells cause graft- versus-host disease and how it subsequently alters the balance of immune responses in the skin. To do this we have developed a mouse model of graft-versus-host disease that closely mirrors the disease seen in male patients who have received stem cell transplants from female relatives such as sisters. For this adult male mice receive radiation treatment to remove their blood and bone marrow cells, which includes all their immune cells. We then inject female bone marrow cells and T cells into the tail vein to replace the missing immune system. Sometimes we use bone marrow from genetically modified mice for these experiments. This allows us to either trace specific cells because they carry a fluorescent marker and/or find the function of a protein by using bone marrow from mice that lack that protein. For the radiation, the mice are placed together in a special box and put into the irradiator which is a dark warm space that they are comfortable in. The radiation dose we use means that they sit in the irradiator for 10 minutes before being put back into their cages. We have carefully monitored the amount of radiation needed to mimic the conditioning treatment that patients need and have found that it works best when the final dose (11Gy) is split and given as 2 radiation sessions of 5.5Gy 2 days apart. This means that the radiation hit each time is less and helps stop problems that might happen due to e.g. damage that happens in the gut. We also put the mice on antibiotics, which are mixed in the drinking water, before the first irradiation. This stops the mice from getting sick because they can't fight infection without an immune system, and also because the radiation can cause leakage of bacteria from the gut. After the second round of radiation, we injected the bone marrow and T cells in the tail vein as described in the paragraph above. Graft-versus-host disease will develop in all of the mice that receive T cells. In most of the mice (80-90%) the disease lasts for about 4 weeks until it begins to resolve and the mice recover. The most obvious sign of the disease is that the mice lose weight. We check them every few days to make sure that this is not excessive which is judged as 20% of their body weight. The mice are also given special soft food in their cages so it is easy for them to eat if they are feeling poorly. While the mice have graft-versus-host disease we check them every few days using a health check system that we know gives a very reliable indication of how sick the mice are. This includes assessing how active the mice are, and whether their fur is sticking up, which is a sign that they feel unwell. Most of the mice some some signs of disease but remain active and begin to gain weight again about 2 weeks after the transplant. A small number of the mice will develop

more severe graft-versus-host disease however, in which case we will kill them humanely before they get too sick.

The other area of work we do in the lab is to investigate immune responses to tumours in the skin. For this the mice will receive injections of tumour cells in the skin (intra-dermal as described above). This is important to make sure the tumours grow in the same place as melanomas would in patients. These tumours grow as a single mass that we can measure over time up to about 15 days. We inject the tumour cells into the skin on the side of the body of the mouse to make sure that as it grows it will not be in the way when the mice move around and carry out daily activities. At different time points we will kill the mice humanely and analyse the dendritic cells and other immune cells in the lab. These mice might receive injections of e.g. antibodies via the tail vein to increase T cell activity to mimic immunotherapy treatments used in the clinic. In this case the mice will be injected approximately even 3 days for up to 2 weeks and we will compare tumour growth to control animals that did not receive the antibodies. Injections of antibodies or other drugs to block different aspects of the immune response will only cause transient discomfort as explained above.

In a few experiments we will use a microscope to see the movement of cells in the skin. Do to this the mice are given a general anaesthetic using an injection so that they are asleep and still while we do the experiment. Typically we use what we call terminal anaesthesia which means that the mice will not wake up from the experiment and will be killed in a humane way at the end. We look at the cells in the ear skin by putting the mice in a special restrainer so that the ear is in the right place for the microscope imaging piece, and record the image on the microscope for about 2 hours. By using the ear skin we do not need to do anything to the mouse to expose the area we are looking at, and because the mouse is asleep them won't experience any discomfort. We can top the anaesthetic up during the experiment if the mouse is coming out of deep sleep and the mouse is placed on a warmed heating mat to make sure they do not get cold during the experiment.

At the end of by far the majority of experiments (>95%) the mice will be killed using humane methods, usually this involves putting them in a dark box attached to a machine that provides controlled levels of carbon dioxide. Under these conditions they go to sleep before stopping breathing. We then make sure they are dead by breaking the join between the spinal column and the brain, known as cervical dislocation. Occasionally (<5% of the mice) we need to get more blood cells from the mice than we can get from tail vein blood samples. In this case we will use an alternative method to humanely kill the mice by using terminal anaesthesia to put them in a deep sleep before taking the blood by putting a needle into the heart (known as cardiac puncture). The mouse will be killed without waking up and so will not experience discomfort or any distress during this method of euthanasia.

What are the expected impacts and/or adverse effects for the animals during your project?

For the majority of healthy animals analysing skin immune cells following an intervention of some kind, mouse health and behaviour is not expected to be altered as a consequence. Because we are studying immune responses in the skin, we frequently apply substances topically, by painting them onto the back of the ear or the shaved body skin. This may happen over a period of 1-2 weeks.

Repeated application of something that causes inflammation could lead to dermatitis. In this event we will stop applying the substance, and kill the mouse by a humane method if the irritation has not improved within 1-2 days.

However, because we are investigating how immune cells function during and after graft-versus-host disease and cancer, we need to cause disease in these mice. This means that our programme has a moderate level of severity. For our graft-versus-host disease model, mice will initially show clinical signs of graft-versus-host disease including weight loss and loss of activity, and will feel unwell (as patients do after transplants) but most of them will recover, gaining weight from about 3 weeks post- transplant. In the lab we have developed a very sensitive scoring system which allows us to monitor the mice closely, and if they are not recovering from graft-versus-host disease or are looking very sick, then we will kill them in a humane way.

For our cancer experiments, we need models in which skin tumours are growing in order to find new strategies to activate the immune system to destroy tumour cells. We make sure the tumours grow in places that do not get in the way of day-to-day activity of the mice (e.g. moving, grooming, eating) and

the mice are monitored to make sure the tumours do not get too big. Therefore, we do not expect the tumours to make the mice feel unwell. In the rare cases where tumours suddenly grow very quickly or break down to cause wounds, the mice will be killed in a humane way. Patients presenting in clinics with cancer ofter have metastatic disease, which is much harder to treat that single primary tumours. For a minority of our cancer experiments (<10%) we will inject tumour cells into the blood of mice to replicate metastases and spread of skin cancers. In these experiments mice may show signs of being sick by losing weight and being inactive. We will use our severity scoring system to monitor these mice closely and kill them if they are very unwell.

For all of our protocols we will use standard techniques such as injection of cells or substances to alter immune responses, and sampling the blood to monitor changes during an experiment. We do not expect these interventions to adversely effect the mice beyond the transient discomfort at the time of injection.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of protocols in this project (3/5) require disease models and as such severities may reach moderate levels for 70% of mice used. 30% of mice will experience a mild severity due to breeding of genetically-modified mice, and limited interventions carried out on healthy animals.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our work focuses on understanding complicated interactions between lots of different white blood cells within a 3-D tissue environment (the skin or tumours). Part of these interactions need cells to be recruited from other parts of the body via blood vessels. Because of this, these experiments can only be done in live animals. In order to achieve our goals we propose to use the mouse as the model organism to study interactions between cells of the immune system in the skin and tumours in the development and function of the immune system for several reasons. Genetic modification of mice is well-established and their blood and immune system has been intensively studied and bears extensive similarities to that of humans. There are numerous reagents that help the study of the immune system, which is not the in other organisms. To our knowledge no other species of lesser sentience can fulfil the requirements of this programme to the same extent as the laboratory mouse.

Mice are the lowest vertebrate group in which well-characterised animal disease models have been developed. Furthermore, the mouse immune system is probably the best characterised amongst vertebrates, and a number of immune models now exist in genetically modified mice that help us to investigate the molecular interactions between immune cells.

Which non-animal alternatives did you consider for use in this project?

There has been a lot of work developing human skin equivalents in other labs and skin organoid cultures.

While we do not think we can completely replace the use of mice for our experiments, we have projects in the lab that are investigating the use of computer models to generate interactive maps of cell behaviours, specifically Langerhans cells and melanomas in the skin. We think that these could be used to predict ways in which Langerhans cells might sense and respond to growing melanomas and identify the most important signalling

molecules before we start experiments in mice. This approach could replace the use of mice for early discovery experiments.

Why were they not suitable?

At the moment scientists have not found a way to add white blood cells to these models, which live and function as they would in the body. These models also do not allow us to study the entry of white blood cells from the blood into the skin or tumours. We will continue to follow progress into these models in order to integrate them into our research in the future.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers of mice are based on our experience with previous projects that use similar experiments. This includes 5000 mice over 5 years fro breeding based on the use of genetically-modified mice which often requires breeding of heterozygous mice to produce homozygotes and wild-type littermates as controls.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will adopt the best possible practice on the principles of experimental design wherever possible and all data will be published following the ARRIVE guidelines. We have received training in the use of the NC3Rs experimental design assistant (EDA) tool and this was used to plan experiments for protocols in this project.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Wherever possible, we use both male and female mice.

Breeding strategies for genetically-modified mouse lines ensure that, where possible, control groups can be taken from littermates or that control and experimental lines are derived from common parentage to ensure background genetic composition is equivalent.

An advantage of working with the skin is that we can obtain a lot of tissue from one mouse, and also other organs may be shared and used. For example, we frequently use non-involved skin from the same mouse for controls. We can also divide skin and tumours so

that tissue from a single experiment is subject to different analyses (e.g. microscopy and flow cytometry).

Lab analyses such as flow cytometry (FACS) and sequencing of expressed genes allows us to generate large data sets from small numbers of mice. These data allow us to generate hypotheses that we can test in larger groups of mice designed with the EDA.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In order to address our experimental aims, we need to use animal modes that allow us to replicate the aspects of human disease that are important to our work. For this project this is the movement of cells into and out of the skin and tumours in the skin, and interaction between these cells during graft- versus-host disease.

We propose to use mouse models for the following reasons:

1) These experimental models are well-established in mice and as such have been optimised to generate the minimal level of disease that still induces the effects that we are studying. For example, in our graft-versus-host disease model, the T cells from the transplant need to be present in sufficient numbers to enter the skin and kill immune cells, including Langerhans cells, therein. But we have titrated the number of T cells so they cause a self-limiting disease that reduces the duration of suffering. Alternatively, for our tumour experiments it is sufficient in most cases for mice to grow a single primary tumour, and we do not need models where this tumour spreads or gets too big.

2) There are a wealth of well-tested reagents for use in mice that mean that we are not testing new drugs with unknown effects that could cause harm and suffering.

3) We have worked with the animal house staff at our establishment under our project licenses for nearly 10 years and have a established an excellent partnership of support and understanding about our models. This means that the staff know when the mice are most likely to feel unwell after receiving irradiation and bone marrow transplants and are careful to minimise distress and suffering, for example by providing soft food and extra bedding in cages of transplanted mice.

Why can’t you use animals that are less sentient?

To our knowledge no other species of lesser sentience can fulfil the requirements of this programme to the same extent as the laboratory mouse. This is because the cells and functions of the immune system are highly conserved between mice and humans. Other model organisms such as flies or zebrafish share some aspect of the innate immune system, but do not have an adaptive immune response. Also, these organisms do not share the conserved skin immune anatomy that is present across mammals, for example the presence of hair follicles.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have a very carefully worked out scoring system in the laboratory (that has been adopted by many other groups which share our animal unit). This recorded monitoring allows us to identify mice that are looking more unwell then we expect from our experiments. Depending on the score, these mice will either be given soft food to help them eat and drink to aid their recovery, or, if necessary killed by a humane method before they suffer too much.

We have a lot of experience with our graft-versus-host disease model and have refined it to minimise suffering for the mice. This includes giving the irradiation as 2 separate doses over a period of 2 days. This reduces damage to the intestines caused by higher doses of irradiation and therefore the mice are healthier. We have also tested the number of T cells transferred to cause clinical sub-lethal graft- versus-host-disease, where most of the animals recover.

By working with the skin, we can often paint a substance onto the skin rather than injecting it into the mouse. This reduces suffering both because we avoid the use of needles for injections, and also because we can induce a local response that does not make the animal feel unwell.

For some of our work we may be able to use inducible genetically engineered models which are more refined because they allow us to investigate the consequences of changing a gene or cell-type at defined time-points.

In the event that any of our animals are unexpectedly unwell, then the vet will be called to advise on how we can minimise suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Use of NC3Rs resources and links within the website provides us with best practice guidance, for example there are guidelines on the use and design of pilot studies (https://www.nc3rs.org.uk/3rs- resources/conducting-pilot-study), breeding colony management (https://www.nc3rs.org.uk/3rs- resources/breeding-and-colony-management), non aversive methods for handling mice (https://www.nc3rs.org.uk/3rs-

resources/mouse-handling) and the single use of needles for injections (https://www.nc3rs.org.uk/3rs-resources/single-use-needles). We also use the guidance by Workman et al on the welfare and use of animals in cancer research (https://pubmed.ncbi.nlm.nih.gov/20502460/).

We will seek to up-date best practice using the NC3Rs website and on advice from the named information officer.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All new members of the lab are encouraged to attend NC3Rs training courses, and use NC3Rs resources to keep us informed about advances. We also benefit from posters and information in the foyers of our animal units.

3Rs approaches are embedded in the ethos of the lab and we are constantly looking at technological advances that would allow us to replace the use of mice with alternative systems.

84. Neural Bases of Action

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Neural circuits, Motor control, Sensory System

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Goal-directed behaviours rely critically on internal representations of the surrounding space. While we understand increasingly well how neurons represent space, how brains construct such spatial representations and use them to guide action remains unknown. Here, we plan to investigate the transformation of neural spatial representations into actions, and how such representations emerge from sensorimotor experience acquired during developmental stages.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The voluntary control of actions is an essential mechanism that allows us to live a purposeful life. We operate it effortlessly in normal circumstances and only become aware of its importance in debilitating neurodegenerative conditions. Our work aims to identify the

key neuronal and molecular mechanisms responsible for the normal function of the action control system.

What outputs do you think you will see at the end of this project?

We believe that our research will improve our fundamental knowledge of movements execution by: a) identifying neuronal populations that are crucial for specific features of motor execution. b) characterising novel aspects of the function and assembly of motor circuits. c) in prospective, long term, identifying and targeting neuronal populations whose impairment leads to the severe motor defects.

Who or what will benefit from these outputs, and how?

Our research will principally benefit our basic understanding of how the nervous system control motor planning and execution. While the focus is on basic research, our discoveries might also have clinically relevant repercussions through the identification of novel therapeutic targets for the treatment of movement disorders.

Within the context of our work we have generated and will generate transgenic mice that will be of use not only to our project but the all scientific community. Within the scope of this work we already generated viral vectors that have already been adopted by the scientific community and will continue to do so. These technological developments have been greatly appreciated by the scientific community and have been the main focus of review works. Our latest work also led to a successful patent application indicating that the output of the work might also have a translational impact of interest to the pharmaceutical industry.

How will you look to maximise the outputs of this work?

Findings will be made available to other scientists through publication in open-access journals and presentations at scientific conferences and meetings.

Species and numbers of animals expected to be used

- Mice: 23700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The role of neural networks activity in motor performance can only be studied in the intact, freely moving animals. Implantation of chronic indwelling electrodes in humans is only permissible in a very small number of clinical situations and thus is impractical for research purposes. Mice are the experimental species of choice because it is possible to generate

and acquire genetically modified strains, which allow the visualization and manipulation of selected neuronal populations. Adult mice will be used to study neural circuits involved in action selection, while neonates and juvenile will be used to study how these circuits shape during critical phases of development.

Typically, what will be done to an animal used in your project?

The advantage of using mice for our project is that we can selectively mutate genes of interest. To this aim we generate mutant animals by injections, for example, of genetic material in eggs followed by in vitro fertilization. To make sure that the animals born from these procedures do indeed carry the mutant gene, we take a very tiny piece of tissue from the outer ear and test the expression of the genes of interest. This causes minimal distress or pain to the mice. Some animals, adults ad neonates, most typically adults, undergo surgical procedures and for this reason we expect them to show clinical signs of a moderate severity as a result of electrodes, fibers or cannulae implantation. Surgeries last about

1-3 hours, during which we will make a very small window in the skull to gain access to the brain. After which, we implant tiny screws and a probe no more than 5 mm long. We finally seal everything with dental cement. They are expected to recover very quickly from the surgery, typically they are already walking around the recovery cage 15-30 minutes after the surgery. To study the visual system, we also perform injection in the eye, the capillary we use for the injection is very small, about 2-3 times the size of a hair. Mice recover quickly and normally show no signs of sight loss. Mice are then assessed in a variety of behavioural tasks. At the end of the experiments mice will be euthanised using a large dose of anaesthetic followed by cardiac perfusion, which allows preserving the tissue for the successive analysis. At no point during the procedure the animal is conscious or feels any pain.

What are the expected impacts and/or adverse effects for the animals during your project?

Surgical mice will experience some discomfort after surgery, and this is expected to last no more than 24 hours. Very rarely after surgery the severity of clinical signs may be such that the humane end points may be reached. Unless otherwise specified, the administration of substances and withdrawal of body fluids will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm.

To assess the behaviour of the animal, mice are kept on a diet so to increase their propensity to perform specific tasks in order to obtain food reward. These diets only take down the weight of the animal of about one tenth of their initial weight. This is only a partial deprivation as a food will still be given daily to the animals, they simply won’t be in a free feeding regime. Since food intake is not restricted during the task, well-performing mice might overall receive the same amount of food that they would normally receive in an ad

libitum feeding regime, however confining the majority of this food intake in shorter period of time.

Body weight will be measured daily. On the rare occasion that an animal approaches 20% weight loss within the food restriction period, then it is given immediate access to additional food.

With respect to the administration of noxious substances, this will lead to temporary irritation of the injected site. The irritation should disappear within 6h of the administration

Localised heat will provoke a transient mild pain for the duration of the stimulation. The painful state disappears shortly after the stimulus is removed <1h. Animals will be monitored at regular intervals. While the intensity is set so not to cause burns, there is still the chance of skin burns.

Animals will be monitored after the tests for sign of excessive persistent protracted licking/scratching of the stimulated sites or presence of sustained skin irritation. Animals will be monitored at regular intervals and a score sheet for pain and oedema will be implemented. Mild cortisone creams can be topically applied if such reactions are noticed. Analgesia can also be given immediately following the test to prevent the occurrence of pain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for the vast majority of the mice (>80%) will be mild as we don't expect to work with animals carrying debilitating genetic conditions. Mice are expected to reach moderate level of severity exclusively during surgery and during the period immediately following the surgery, which represents <2% of the time spent by the animal in this protocol.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The role of neural networks activity in motor performance can only be studied in the intact, freely moving animal. This is because network activity depends on the communication of

multiple, distant brain areas and incoming sensory stimuli. Hence, we can only uncover the network dynamics in vivo.

Which non-animal alternatives did you consider for use in this project?

We have collaborated (and will continue to do so) with colleagues who devise computational models of motor networks and have on occasion used these to design experiments and predict their outcomes.

We have also considered the possibility of studying these aspect directly in humans.

Why were they not suitable?

With regard to carrying on these studies in humans, the implantation of chronic indwelling electrodes in humans is only permissible in a very small number of clinical situations and thus is impractical for research purposes. There is no non-invasive method of monitoring the firing patterns of groups of individual neurons in humans. Human neuroimaging techniques (fMRI, PET, MEG) lack either fine temporal resolution (PET and fMRI) or fine spatial resolution (MEG), and anyway deal with comparatively large sections of cortex. They cannot provide information about individual neurons.

With respect to computational models, these are still extraordinarily simple in comparison to the complexity of the brain, and cannot substitute for experiments themselves.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Husbandry: In order to pursue the key objectives of this license we will need to make use of transgenic animals. We estimate that over the course of the years we will need to make use of about 50 distinct transgenic lines. These includes mostly but not exclusively: population specific CRE and FLP mouse lines, which will be used to identify genetically defined neuronal populations; various versions of reporter lines, which will be needed to visualize those neuronal populations; activity modulators that will be crossed to FLP and CRE lines in order to alter the activity state of the identified neuronal populations. The establishment of those colonies will require, based on our previous experience, about 2075 animals.

Breeding and maintenance of the established lines is estimated to require, based on our recent experience, around 17000 animals for the duration of the licence. This is taking into

account the need of refreshing any given running line about 5 times per year and an expected usage of about 30 distinct mouse lines per year. We plan to maintain for any running lines an average of 12 females and 6 males.

These numbers ensure a smooth work flow for 4 lab members in order to provide access to the lines also in the case of parallel work with the same lines of multiple members (a situation that will occur frequently for the most commonly used lines, such as reporters' lines).

Experimental testing (e.g. electrophysiology, imaging, tracing, behaviour): Below we provide estimates, with justifications, for the number of mice required to test the family of hypotheses raised in each work package. While not exhaustive of all described experiments this paragraph presents the statistical reasoning behind four specific examples that capture the key features of proposed work.

Outline of the classes of hypotheses: Broadly, these hypotheses revolve around whether or not populations of cells are similarly/differently tuned to certain features of the movement in wild type animals (A) and genetically defined neurons (B); similarly, we wish to determine whether the neural networks upstream of these populations are both spatially non-overlapping (C) and/or functionally distinct in their tuning e.g. to sensory stimuli (D).

In considering tuning to features of the movement in WT (A), our previous experiments suggest that n=8 mice per collicular site for tetrodes recordings, and n=12 mice per site for the optogenetic experiments, are enough to reveal a statistically significant effect if it exists. Given our ambition to map a matrix of 3x3 collicular sites, and our combined historical success, we estimate around ~90 mice will be required for the optrode recordings and a further ~20 mice for the combinatorial stimulations experiments using custom-designed optic fibre arrays.

For tuning to features of the movement in genetically defined populations (B) (our preliminary results indicate a yield of tuned genetically defined units of ~5-10% of all recorded units depending on genotype. We estimate the need of recording ~10 mice per site per genotype. We aim to characterise at least 2 genetic types (inhibitory-pre and inhibitory-post) for the inhibitory population, a general inhibitory population and a general excitatory population. Given the initial exploratory phase for the search of the correct inhibitory populations, we estimate ~150 mice to characterise three distinct SC domains. Numbers will scale as with the number of genetic lines to be tested

For the spatial organization of brain-wide networks (C): our null hypothesis is that the sets of cortical inputs emanating from a given area of interest (AOI) converging onto any two distinct collicular glomeruli are spatially indistinguishable. Our experiments will consist of viral injections in such glomeruli pairs, aimed at revealing the spatial distribution of their cortical inputs. Due to the finite spatial resolution of viral injections, paired injections will need to be performed on a coarse 3x3 grid of 9 collicular sites at most (the same sites in all mice), and replicated three times to validate consistency. Among the 36 distinct pairs of

glomeruli/sites, we will investigate those that are two grid points away from each other, i.e. 8 pairs. For each pair, we shall test the null hypothesis by projecting the topographical locations of all labelled presynaptic soma for each of the two sites, onto the axis that maximally discriminates between them. Based on our experience with viral-based tracing, we estimate that we will exceed the necessary discrimination-threshold of 1000 labelled neurons in only about 1/3 of our attempts.

With respect to functional sensory tuning specificity (D), we shall follow a similar approach, now testing the null hypothesis that the cortical inputs to distinct glomeruli have no distinguishable tuning to sensory stimuli of the relevant modality. Although no research group has ever traced the cortical input to defined collicular neurons, let alone their tuning, we can roughly estimate the typical spread in preferred orientations for visually-tuned neurons in the superficial layers of the superior colliculus.

From this, we can caluclat with the appropriate statistical methods that ~80 tuned neurons per module are required. Based on previous studies in the V1 of awake mice, we may expect 30% of all labelled neurons to be tuned to the stimulus features of interest (e.g. orientation), meaning that we need to label at least 270 neurons. Based on our experience with viral-based tracing, we estimate that we will obtain this number in about 2/5 of our attempts. Thus, we require 8 (pairs) * 3 *(replications) * 5/2 (inverse success rate) = 60 mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

With regard to the husbandry requirements, we leveraged on the data accumulated in our previous licenses that consisted of very similar workflow. With regard to the experimental work, thanks to the work in our previous license we have a good idea of our expected data distributions and we could build wherever possible accurate power calculation models.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Generally we run pilot studies to explore the parameters of an effect before the final design is decided upon. Whenever a non-standard experimental design will be required, statistical advice will be sought.

Whenever possible we make use of ex vivo recordings, this will reduce the instances in which we have to perform in vivo acute or chronic recordings, which greatly decreases the number of animals used under these protocols. However, in most of these cases we still rely upon a phase of in vivo work and rarely on behavioural training prior to the ex vivo work. In these cases we will adhere to ARRIVE guidelines.

Cryopreservation will be used to preserve important lines and remove the necessity to hold stock for extended periods. On occasion we may need to ensure that our protocols are

optimised and this may require the re-implantation of un-manipulated oocytes, embryos or blastocysts. Cryopreservation of embryos and sperm will be used for long-term storage of genetically altered mouse lines and pedigree lines with in vivo viability assessed to ensure that lines can be re-established successfully.

Rederivation will be undertaken should the health status of the animals be compromised in a way that would significantly affect the welfare of the animals or where the experimental results might be altered unduly.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the species of choice in most areas of biomedical research, allowing us to use powerful techniques such as transgenic animals and knock in/knock out, which would not be available for other species. For instance, the mouse is the only animal for which transgenic strains are currently available that allow us to genetically identify neuronal populations that would be otherwise undistinguishable.

This is crucial for the success of our project, as it relies on assigning individual function to identifiable neuronal cohorts.

The surgical approaches used are the least severe available, involving the smallest amount of tissue damage. Animals are given extensive post operative care including analgesics. Animals are closely monitored throughout the experiments and any signs of problems with implants or other aspects of surgery are immediately dealt with, or, if this is not possible, the animal will be culled. Similarly, animals are closely observed and monitored during the recording experiments and during interactions with other animals. All surgical procedures are carried out with the best possible level of asepsis and according to best practice (internal guidelines).

Prior to behavioural testing animals might be subjected to a dietary restriction of food. These restrictions are required in order to have the animal engaged in the task, in particular when multiple choices are presented and one has to be reinforced. Based on our previous experience the exclusive presentation of a reward is not effective in mice in absence of a dietary restriction and the animal is incapable or unwilling to perform the task. In a typical behavioural session, the animal is unrestrained moving in an open field

and can be presented with multiple water or food delivery ports or delivery sites and it has to reach one of them in order to obtain the reward. Reaching movements might involve limb or head/body movements to the target. Assessing animal behavior is key to determining whether or not the brain regions and neuronal populations we study are indeed involved in the planning and execution of spatially directed movements and hence an essential part of this programme.

In a separate behavioural testing paradigm, we will investigate the ability of the animal to identify the location of a noxious sensory stimulus (e.g. mild pain). In order to produce a very localised noxious stimulus, we will use either a localised source of heat or the injection of small quantities of formalin and then test the ability of the animal to identify the location of the stimulus under different experimental conditions and different genetic backgorunds.

Why can’t you use animals that are less sentient?

The mouse is one of the simplest mammals in which to study neural control of motor function. The role of neural networks activity in motor performance can only be studied in the intact, freely moving animal. Implantation of chronic indwelling electrodes in humans is only permissible in a very small number of clinical situations and thus is impractical for research purposes.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Every step of our work has been carefully assessed so to minimise welfare costs and it's constantly re- assessed as improved methods become available. These steps include:

- post-operative care: e.g. our animals are hosted in a temperature/humidity regulated cabinets and monitored post-operatively by qualified staff.

- pain management: e.g. together with out vet we assess and implement the most effective systems for pain management pre, during and following surgical procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the Home Office Guidance on the Operation of the Animals and NC3Rs guidelines, specifically the ARRIVE guidelines(Kilkenny et al., 2020).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our Biological Services Group and NIO keeps us constantly informed with advances in the 3Rs, we follow the NC3Rs newsletter and their seminars and demonstrations. We

constantly try to refine our techniques and standardise them once these bring tangible improvements.

85. Investigating the Regulation of Energy Metabolism

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

AMPK, Cancer, Lipids, Metabolism, Obesity

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

AMP-activated protein kinase (AMPK) is an enzyme that plays a major role in regulating cellular energy metabolism. The overall aim of this project is to gain a better understanding of how AMPK regulates metabolism and how this regulation is altered in certain diseases, such as obesity and cancer. An important aspect of the work is to determine how modulating AMPK activity either genetically or pharmacologically impacts on disease progression in pre-clinical mouse models.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

AMPK is widely distributed in our body and acts as a master switch to regulate the energy metabolism in our cells. Changes in cellular metabolism are known to play an important role in a number of human diseases, including two of the most common health problems in the world, namely obesity and cancer. However, the underlying mechanisms that drive these changes remain poorly understood. It is important to carry out our scientific work in order to increase our understanding of the precise causes that lead to dysregulated metabolism. This will help us to design ways to prevent or slow disease progression. These could include both changes in lifestyle e.g. diet, or pharmacological approaches, targeting key proteins involved in metabolism. Additionally, a greater knowledge of the key molecules that drive altered metabolism might themselves provide new biomarkers for predicting disease progression and/or diagnosing disease.

What outputs do you think you will see at the end of this project?

The outputs of the work will include:

1. Increased understanding of the regulation of energy metabolism and how this contributes to diseases such as obesity and cancer.

2. Identification of new targets for drugs aimed at the prevention and/or treatment of these diseases.

3. Characterisation of new markers to diagnose and evaluate disease progression and/or monitor the efficiency of treatment.

4. Written information such as publications available to other researchers and the public to increase their understanding of energy metabolism.

5. Presentations at scientific meetings and public engagement events.

Who or what will benefit from these outputs, and how?

In the short-term, our work will benefit:

1. Biochemists: they will increase their understanding of the fundamental role of AMPK in energy metabolism;

2. Biologists: they will increase their knowledge in how AMPK is involved in metabolic diseases such as cancer and obesity;

3. Our current research: outputs from the work such as publications and public engagement events will help us to get further funding to continue our research and for training early-stage scientists in metabolic research.

In the long-term, our work will benefit:

1. Collaborative opportunities: if AMPK is defined by our work as a pivotal factor in obesity and cancer, then collaboration with pharmaceutical companies will lead to the design and testing of new drugs.

2. Clinicians: clinical trials using new drugs can be undertaken. Our research will also enrich the knowledge of clinicians in treatment of obesity and cancers. In turn, this will help us in recruiting patients for future clinical studies.

3. Patients: if clinical trials are successful and new drugs are approved for clinical treatment, patients suffering these diseases will benefit. Importantly, their families will receive indirect benefit from our work that will help improve their quality of life.

4. The Economy: effective treatment of obesity and cancer will reduce the financial burden of the UK health service and/or worldwide.

How will you look to maximise the outputs of this work?

We will use a number of approaches to maximise our outputs:

1. Collaboration with other groups to bring new expertise, access to new reagents and/or new technologies that we do not currently have ourselves.

2. Collaboration with industry when our work reaches the translation stage. Our institute has excellent resources for helping researchers to establish collaborations with industry.

3. We will use a variety of approaches to disseminate our findings, including the traditional routes of journal publications and conference presentations, but also including social media threads, podcasts and interviews.

4. For approaches that report null results, we will deposit our findings on preprint serves, as well as discussing them at scientific meetings to ensure that the information is accessible as widely as possible.

5. We will also publish methodological papers in addition to papers describing our scientific findings, in order to move the whole field forward and increase the rate of progress.

Species and numbers of animals expected to be used

- Mice: 17000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice for our studies. Mouse models have been widely used to study metabolism and to model human diseases, e.g. obesity and cancer, and are the lowest mammalian species available for such studies. In addition, the mouse is the most tractable mammalian species for genetic manipulation. The techniques required for generating genetically modified mice have been extremely well characterised and provide a robust and reliable approach for developing new mouse lines for studying metabolic regulation. Most of our studies will use adult mice as we want to understand long-term changes in metabolism and related disease situations. For some studies, we may use juvenile mice (under 8 weeks of age) to study the impact of altering diet at an early stage e.g. beginning a high-fat diet study on mice aged 4-6 weeks. This will allow us to understand the effect of altering diet before the mice have reached the adult stage.

Typically, what will be done to an animal used in your project?

Wild type and/or genetically modified mice will experience the specific procedures according to the disease categories (obesity or cancer) being studied.

1. Mice required for the study of metabolism and obesity will typically have the below procedures done on them.

We will feed mice either with standard chow diet or altered diets (e.g. a diet high in fat) and monitor their metabolism. To monitor their metabolism, we would record their food intake (once or twice for several days during the course of the study), record their body weight weekly, and take blood samples to measure blood glucose levels monthly. We would also use a special instrument called an echoMRI machine to measure their body fat composition (two or three times to during the course of study). The data from these monitoring experiments will help us to determine how the mice have responded to the altered diet.

In some cases, we will use wild type mice that are treated with a specific drug to target a known enzyme in order to alter metabolism. In this case, we may administer the drug using a special type of pump, usually inserted just beneath the skin, in a process requiring minor surgery.

In all cases, if we see a response, we would measure the respiratory rate of the mice using specialised home cages (typically for 5 days, once, possibly twice during the study). These specialised home cages record the volume of carbon dioxide the mice produce as well as the, volume of oxygen they consume. This allows us to calculate the calories they burn, indicating their metabolic rate. In some cases, we may implant small, specialised probes which allow us to measure physiological parameters over time, e.g. blood glucose, blood

pressure, core and/or localised body temperature, heart rate. We need to use surgery to implant these probes and this would be carried out under anaesthesia. For studies requiring implantation of these probes or the specialised pumps, we would usually restrict surgery to a single procedure and would remove the devices on termination. The total duration of all of the combined studies mentioned above would normally be 3-9 months. At the end of the study, mice will be humanely killed and tissues removed for subsequent analyses.

2. Genetically altered mice bearing cancer models (e.g. prostate cancer or liver cancer) will undergo the following procedures:

Typically, we would not routinely monitor their whole-body metabolic phenotypes, so would not normally carry out any specific procedures (other than monitoring the mice for signs of adverse health). At the end of the study, mice will be humanely killed and tissues removed for subsequent analyses.

In some cases, we may change the diet of the mice, e.g. from a standard chow diet to a high fat diet (for up to approximately 3-4 months). In other cases, mice might be treated with or without a drug, administered either by oral gavage or in the food or water (for up to approximately 3-4 months). The total duration for these studies would typically be between 4-12 months. At the end of the study, the mice will be humanely killed and tissues collected for subsequent analyses.

3. Implantation of cancer cells into mice

In some cases, we will study tumour growth by injection of cancer cells either under the skin into the flank of the mouse or into the site where the cancer would normally grow e.g. the prostate. In this case, mice would require a surgical procedure to inject the cells into the tissue. Following injection (either under the skin or into the tissue) the mice would be treated with or without a drug, administered either by oral gavage or in the food or water or through surgically implanted pumps. Tumour growth would be monitored during the study either by physically measuring the size of the tumour with calipers (for tumours under the skin), palpation (for prostate tumours), or using special imaging approaches that allow the tumour cells to be visualised with a modified camera. Typically, the duration of the study would be 4-12 weeks. At the end of the study, mice will be humanely killed and tissues removed for subsequent analysese.g. to determine whether metastasis has occurred.

4. Implantation of adipose cells into mice

In some cases, we will study the effect of transplanting adipose cells (adipocytes) under the skin into the flank of the mouse. The transplanted adipocytes will be isolated either from other mice, or in a limited number of studies, from human subjects. Once transplanted, we will monitor the mice in order to determine whether the adipocytes alter the metabolism of the mouse. In some cases, this will mean feeding the mice altered diets

(e.g. a diet high in fat). Typically, we would record their food intake (once or twice for several days during the course of the study), record their body weight weekly, and take

blood samples to measure blood glucose levels monthly. We would also use a special instrument called an echoMRI machine to measure their body fat composition (two or three times to during the course of study). The data from these monitoring experiments will help us to determine how the mice have responded to the altered diet. If we see a response, we might extend our study to measure the respiratory rate of the mice using specialised home cages (as mentioned in point 1 above). Similarly, as mentioned in point 1, we may implant small, specialised probes which allow us to measure physiological parameters over time, e.g. blood glucose, blood pressure, core and/or localised body temperature, heart rate. We need to use surgery to implant these probes and this would be carried out under anaesthesia. For studies requiring implantation of these probes or the specialised pumps, we would usually restrict surgery to a single procedure and would remove the devices on termination. The total duration of all of the combined studies mentioned above would normally be less than 6 months. At the end of the study, mice will be culled and tissues removed for subsequent analyses.

What are the expected impacts and/or adverse effects for the animals during your project?

In general, for the majority of animals we use, we do not anticipate any significant adverse effects. Animals undergoing invasive procedures such as surgery are expected to make a quick and full recovery.

In some mice, the expected impacts and/or adverse effects might be:

1. Hight fat diet may cause a greasy coat to mice, but this won’t irritate them. In rare cases, this may lead to slight skin damage which is treatable.

2. Diet alteration such as fasting and restricted food intake may cause temporary weight loss and we will control the loss to no more than 20% of their starting weight at the point of the procedure.

3. In some studies, we will be using a genetically modified mouse strain that shows progressive obesity from 3-4 weeks of age. These mice may show clinical signs such as laboured breathing. They also have impaired wound healing and hypothermia. Wounds can be treated and hypothermia can be controlled by supplying extra bedding. Laboured breathing will be an indication to stop the study.

4. In some studies, we will use a genetically modified mouse that can sometimes lead to a degree of cardiac hypertrophy which may cause laboured breathing, and can on rare occasions result in sudden cardiac arrest, particularly when under stress, in mice older than 6 months.

5. In some studies, we will use a genetically modified mouse strain that pre-dispose the mice to certain cancers, such as prostate and liver. However, for our studies, these cancers are subclinical and therefore are unlikely to show any adverse health signs such as pain or excessive weight loss (more than 15% of their initial body weight).

6. In some studies, we will use mice that have a compromised immune system (immune deficient mice). Although these mice will be housed in sterilised cages and maintained on sterilised bedding/food/water, they may develop infection and we carefully monitor the mice for any adverse health signs.

7. In some of our studies, we may administer drugs, e.g. tamoxifen that may result in local inflammation around the injection site and/or cause reduced food or water consumption leading to weight loss or dehydration. We will monitor mice for these adverse effects and if they cannot be treated and persist for more than 72 hours we would terminate the study.

8. Some studies require the mice to be singly-housed e.g. monitoring food intake, measurement of respiratory rate, and in these cases environmental enrichment will be provided to encourage normal behaviour. For all studies requiring single housing, experimental plans will place such studies towards the end of the protocol, so that subsequent single housing will be kept to the minimum period possible to complete the experiment.

It is important to note that in all the instances described above, we will closely monitor the expected adverse effects and take suitable actions to either control the adverse effect within their limits or terminate the mice, therefore the duration of the adverse effects will be minimum.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All mice used within this license are expected to fall under the sub threshold, mild or moderate severity categories. Based on our experience and the experiments planned we expect these to be divided as follows:

Sub threshold: 45%
Mild: 15%
Moderate: 40%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals to achieve the aims of our work for several important reasons, outlined below:

1. One aim of our work is to study obesity, so we need an obese model. In humans, the main cause of obesity is due to the increase in consumption of high-calorie foods, rich in fat and sugar. Therefore, we need to study an animal model that eats a similar diet to become obese, so that we can study ways to treat and reverse the obesity.

2. Another aim of our work is to study cancer, including prostate and liver cancers. It is very difficult to carry out research on humans, and so we need to use pre-clinical mouse models in order to validate new targets that can be progressed to subsequent studies in man, including clinical trials.

3. In order to study energy metabolism, we need to precisely measure metabolism by monitoring oxygen consumption and carbon dioxide production to determine the amount of calories burned. These valuable data cannot be collected from non-animal studies such as cells in culture, necessitating the use of animal models.

Which non-animal alternatives did you consider for use in this project?

We have considered using cell-based approaches. For some of our cancer-related studies, we will use human patient-derived cancer cells. These human cells offer an alternative approach to the mouse models. However, there are some serious limitations for their use, such as the relatively short time frame (24-72 hours) available for experimental treatment, meaning that they cannot fully replace the animal models.

Why were they not suitable?

Cell-based approaches are useful for some studies and can give important results regarding the effect of altering specific enzymes on metabolism. However, it is not possible to recapitulate all of the effects of interactions between multiple cell types e.g. hormonal responses, in cell-based models, and so information regarding inter-organ communication can be lost. Similarly, metabolic changes that occur in a specific cell type can have a dramatic influence on immune responses and these can also be lost in cell-based models. Moreover, some of the readouts that we are interested in measuring e.g. food intake, cannot be studied in isolated cells.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This number is based on the projected number of mice needed for our project aims and takes into consideration our experience of previous studies that have been conducted under our previous Home Office Project Licence in which we used similar experimental approaches and similar mouse models.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The choice of genes that we will study is based on previous cell based and in vitro biochemical approaches. Only genes encoding proteins that have been shown to be involved in regulating energy metabolism in other experimental systems will be examined. Although these in vitro studies cannot predict the effect of manipulating the gene in living organisms, they provide valuable clues enabling us to prioritise our experiments and keep animal numbers to a minimum. In designing our experiments, we plan to obtain the maximum possible data from a single animal. All studies are designed with careful statistical considerations with respect to sample size, utilise strategies to minimize bias such as blinding and randomisation and involve precise and reproducible assays which together ensure that the information we gain is robust while using the minimum number of animals. As well as drawing on our significant prior experience and published work, we have consulted the NC3Rs experimental design assistant allowing us to calculate the appropriate sample size based on each experiment. This will ensure that we are using the fewest number of animals and that each animal is being utilised to its full potential.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will take the measures listed below to optimise the number of animals in our project:

1. When breeding genetically modified mice we will use strategies to maximise the use of offspring wherever possible.

2. For physiological investigations, whenever possible procedures will be combined sequentially, and longitudinal studies on the same mice (including non-invasive imaging and serial phenotypic studies) will reduce the overall numbers of mice required to reach the scientific end-points.

3. We will always strive to use the most refined techniques available in order to reduce animal numbers through improved accuracy of measurements.

4. To reduce animal usage, where it is anticipated that a genetically modified line will no longer be of immediate interest embryos may be harvested and stored (frozen) to reduce the need to continue breeding.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice, both wild type and genetically modified. We will study mice with loss and gain of gene function mainly in specific cell types, although in some cases we will study mice with global genetic changes. Targeting specific cell types minimises the chance that genetic manipulations themselves have broad effects on animal well-being. In most cases, the models we use cause no pain, suffering, distress or lasting harm from the genetic manipulations themselves. We need to use genetically modified animals with adverse phenotypes as these are the only models available for our studies and/or alternative models have similar or worse phenotypes. However, we will restrict our use of these models to the minimum number needed for completing our studies. In addition, we will establish and use monitoring and scoring systems relevant to the physiological system(s) affected by the modification, and endpoints will be established. The inclusion of inducible gene targeting is a further refinement as the genetic alteration can be restricted to adults following viral or chemical e.g. tamoxifen, treatment, thus bypassing any potential detrimental effects of genetic alteration on development. In some of our studies we will use interventions e.g. administration of drugs to target key metabolic pathways. In most cases we use established treatments that do not themselves cause pain, suffering, distress or lasting harm.

Dosing regimes will adhere to good practice guidelines and will follow our previous experience using similar strains and/or available literature using the same or related mouse strain. LASA guidelines will be adhered to and the most refined method will be used. Local AWERB guidelines for administration of tamoxifen and doxycycline will be followed. Doses and duration of dosing will not exceed those reported to have the desired scientific effects in the literature. For tumour progression, mice will be monitored regularly. The stages of tumour development that we study are subclinical, with no overt clinical signs expected. Advice from the NVS / NACWO will be sought should any unexpected adverse effects arise. Immune deficient mice (such as athymic nude or NSG mice) may be

used in tumour studies. To reduce risk of accidental infection these mice will be housed in sterilised cages and maintained on sterilised bedding/food/water.

Some mice may undergo recovery surgery. However, appropriate anaesthesia and pain relief will be given to the mice to reduce pain and stress during surgery and post-surgery. Pain, suffering, distress and harm will be monitored following the surgery, and appropriate actions such as treatment for wound infection or termination will be taken to minimise suffering.

Why can't you use animals that are less sentient?

We study metabolic regulation in mice with a view to understanding the potential causes of metabolic diseases such as obesity, diabetes and cancer in humans. In order to carry this out we need to study free-living animals with a similar level of complexity and metabolic regulation seen in mammals and humans. Thus we cannot use species that are less sentient than mammals. In some cases, we need to undertake studies on the same animals over time to see how their metabolism changes, and so these studies cannot be done in terminally anaesthetised mice. In other cases, anaesthesia has effects on the metabolic pathways that we want to study, and would therefore interfere with the measurements.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will take the measures listed below in order to refine the procedures to minimise the harms for the animals. Different measures will be applied depending on the animal experience.

1. Although we anticipate that the majority of our studies will not harm the animals in any way, where there is no prior knowledge of the outcome of the intervention (for example if a new genetic model is being used) and may have an adverse effect, pilot studies may initially be carried out on a small cohort of animals to determine appropriate dose rates/treatments/end points, being informed wherever possible by published data. Animals being used in such pilot studies will be closely monitored for signs of adverse effects and action taken to minimise suffering.

2. Anaesthesia, where used, will be of depth sufficient to prevent the animal being aware of pain arising from the procedure. All surgical procedures will be performed using aseptic, sterile surgical techniques and will be carried out by trained competent personnel reducing the possibilities of post- operative complications and mortality. Mice will be kept warm and monitored regularly until they have recovered from the anaesthesia. Peri- and post-operative analgesics may be administered to alleviate pain or distress for surgical procedures and other procedures which may cause more than transient pain. In all cases, protocols involving surgery will be agreed in advance with the NVS. The grimace scale will be used post operatively to aid in the assessment of presence or absence of pain. The use

of antibiotics following surgery will be administered where appropriate, as agreed with the NVS. Experimental protocols, including dosing and sampling volumes and frequencies, will be in accordance with current best practice (using sources such as the NVS, NACWO, NC3Rs, LASA).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow government guidelines such as the Guidance of the Operation of the Animals (Scientific Procedures) Act, the ARRIVE guidelines, LASA guidelines, the relevant published literature on studying metabolism in mice as well as the PREPARE guidelines, NC3Rs website and locally published guidelines from the 3Rs advisory group of our establishment.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will consult with resources such as the NC3R database and the 3Rs advisory group at our institute. We will consult regularly with colleagues, animal technicians and vets about best practice and potential further refinement of our procedures. Our Institute has recently appointed an Animals Governance Officer and we will liaise closely with this post to ensure we maintain best current practice. Additionally, we will keep up to date with the scientific literature to follow the published best practice in monitoring and assessing mouse metabolism in vivo.

To effectively implement the 3Rs in our work, we will discuss these issues in our weekly group meetings so members of the group who carry out animal work are kept informed to maintain best practice. One role of the animal facility governance is to implement the 3Rs to the whole institute, so we will closely work with this person and work on this person’s feedback on implementation of the 3Rs. We will encourage members of the group to take part in internal and external competitions on 3Rs, which will be a very effective way to implement the 3Rs in our work.

86. Immunity, Resilience and Repair

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Immunity, infection, cancer, healthy ageing

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We want to understand how lymphocytes interact with other cells types to promote immunity, resilience and repair at the organismal level. To achieve this we will combine physiological, cellular, molecular and computational approaches across the life course.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The immune system is now understood to be a collection of distinctive cell types that mediate immune effector functions, such as antibody production or cellular cytotoxicity, to promote immunity. However, the molecular basis for this, including the genes that promote the durability of immunity to infection and limit the effector functions of immune cells to avoid harmful reactions against self and promote resilience remains to be understood. The molecular and cellular basis of how immune cells promote the healing and repair process is essential to understand how these mechanisms operate optimally and deteriorate as organisms age and will be fundamental to treating the common human diseases of the 21st century.

What outputs do you think you will see at the end of this project?

We anticipate the principal outputs will be scientific publications. The intellectual property we develop may underpin the development of new therapeutic modalities.

Who or what will benefit from these outputs, and how?

Other Researchers:

Our work will create unique research tools, methods and data resources relevant to the development and function of lymphocytes. Through publications, presentations and the use of data repositories our work will influence other researchers, although this may not be fully realised until completion of the project.

Industry:

Our previous work on immunomodulation has a proven track record in translational outcomes and successful working with partners in small and large commercial enterprises. We anticipate continuing impacts and our findings will be commercialised, where possible, through collaboration with industrial partners.

Patients and Clinicians:

We will continue to ensure that our research models are relevant to prevention and treatment of human disease.

How will you look to maximise the outputs of this work?

We will continue to publish our findings in peer-reviewed journals making use of open access, preprints and journals that accept negative findings or replication studies.

We will collaborate with colleagues in Universities, Medical Centres, other Institutes and the commercial/ biotechnology sector to promote awareness and influence the research direction of others.

We will deposit datasets and analytical tools in repositories and promote data accessibly through careful annotation, metadata and data visualisation applications.

We will share our research tools and know-how freely.

We will protect intellectual property and patent findings when appropriate.

Species and numbers of animals expected to be used

- Mice: 108070

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse immune system is extraordinarily similar to the human. While there are differences, these are far outweighed by the similarities; the fundamental appreciation that the cells and genes that regulate immunity in the mouse overlaps substantially with humans has brought fundamental health benefits in a global scale e.g., vaccination, skin grafts, therapeutic monoclonal antibodies, cell therapies. Remarkable discoveries such as regulatory T cells and subsets of innate lymphocytes were first made in mice and then found in humans. B and T cell receptors for antigen are formed by similar molecular processes and their repertoires selected and maintained by similar mechanisms.

The mouse has a long history of contributing to fundamental and applied immunology. The ability to delete individual genes in mice - in specific cells, at specific times - enables studies that are not possible in humans. The ability to perform infections or study the growth of defined tumours and take tissue samples for research is impossible or impractical with humans. Studies of controlled ageing cohorts under defined and constant environments are also impossible for researchers to perform over the human life-course.

Typically, what will be done to an animal used in your project?

Female mice (<2000 animals) will be injected with hormones to increase the production of embryos (superovulation) after which they will be killed to recover embryos (Protocol 1). To establish new mouse strains a small number of female mice (<500) will receive embryos via surgical or non-surgical procedures (Protocol 2). A very small number of male mice (<20) will be vasectomised to produce pseudo-pregnant embryo recipients (Protocol 3), these males will be kept until twelve months of age.

We will breed and maintain genetically altered (GA) mouse strains for experimentation. The maintenance until a maximum of 15 months of age will cover the mice used in this study under Protocol 4 where no or rare (<10%) mild effects are anticipated (< 84000 procedures). The majority of mice of both sexes will be studied either after killing by a humane method to address questions on lymphocyte homeostasis, or under the

experimental protocols below to study lymphocyte responses. We will recover post-mortem tissues and purify and/or grow lymphocytes for use in the studies below.

Some GA mouse strains may manifest moderate clinical signs as a consequence of the alterations to their genomes. Protocol 5 (< 150 animals) enables us to address questions of immune homeostasis in mice that may develop clinical signs that exceed mild limits of protocol 4 such as marked piloerection, intermittent hunched appearance, abdominal distension. We have a good understanding of the age of onset of clinical symptoms and when clinical features arise we shall aim to euthanise mice to recover tissues within 24 hours of symptoms being reported, or before exceeding moderate severity, to avoid suffering. Some mice with conditional Myc expression, a PI3K E1020K mutation or BCL6 transgenes may develop lymphoma. We have designed our breeding programme to minimise the numbers of mice that have an oncogenic combination of transgenes. We freeze bone marrow from these mice to use as donor cells for transplantation experiments under protocol 10 to generate a cohort of experimental animals in which the onset of disease is more synchronous.

A minority of mice including inbred strains with no genetic modification with no or mild phenotypes will be aged beyond 15 months (no more than 1300) to enable us to study changes in immunological processes with age (Protocol 6).

One important experimental approach we use, common to several of the following protocols is to use inducible gene deletion. Tissue specific and temporal gene regulation in cells can be achieved using a number of genetic alterations. These studies will involve a minority (~15%) of all procedures.

For the majority of our studies of immunity we will challenge the immune system using non-replicating antigens which trigger lymphocytes through their antigen receptors together with adjuvants or other immunomodulators (Protocols 7: 16,900 animals 7 and 8: 2000 animals).

The distinction between these protocols is that Protocol 7 will use genetically altered mice that have been bred specifically for the protocol, or mice into which we have transferred cells (adoptive transfer) to study how well they perform their specialised functions and how they influence other cells in the host; while Protocol 8 will use mice that have been prepared by stem cell transfer into irradiated recipient mice. The fewer than 2000 mice studied under Protocol 8 need 8-12 weeks to reconstitute the immune system before they can be used for studies of homeostasis or immunity.

When we use infectious agents that replicate, we will prioritise the use of attenuated bacterial or viral stains, live vaccines, or expose to doses of infectious agent from which we expect the animals to recover after experiencing moderate severity. This may result in some discomfort similar in duration and severity similar to that of a vaccination or infection, but is rarely found to lead to severe outcomes.

Under Protocol 9 mice will be inoculated with tumours which will grow for up to two weeks before administration of immune cells and subsequent study of the effect this has on tumour growth. These experiments should not last longer than 32 days and the numbers of animals used will be 500.

The study of the development of lymphoma will be conducted under Protocol 10 (< 400 animals). Frozen bone marrow or other sources of stem cells will be used to reconstitute the haematopoietic (blood cell) systems of irradiated mice. These will be monitored until the onset of clinical signs which is usually within six months of reconstitution.

In order to be able to investigate the ability of T cell subsets to regulate a graft versus host (GvH) response, Protocol 11 will use a proven allograft model using T cell-depleted bone marrow from C57BL/6 mice transferred into lethally irradiated Balb/c recipients and co-transfer of T cell subsets to elicit an immune response (<300 animals). These experiments will typically last less than 60 days.

What are the expected impacts and/or adverse effects for the animals during your project?

For most of the mice, including immunodeficient strains, we do not expect any impacts or adverse effects in our high-quality AAALAC accredited specific pathogen-free (SPF) animal care facility. NOD SCID Gamma (NSG) immunodeficient mice mice can exhibit progressive hearing loss that can be profound at three months of age.

Embryo transfer and vasectomy are surgical procedures with short term post-surgical pain. Post- surgical pain will be controlled by giving pain relief and any animal not fully recovered (eating, drinking, return to normal behaviour) within 24 hours will be euthanised.

For some GA animals bred on this licence there will be adverse events associated with loss of immune homeostasis. These can manifest as lack of weight gain, or even weight loss. Other visible impacts of inflammation include intermittent hunching, piloerection, deterioration of coat condition, a reduction in activity, abdominal distension, scaly/scurfy skin on ear, around eyes and on tails.

Although ageing is a major risk factor for adverse effects, we know that the vast majority of our aged mice remain healthy throughout the duration of their lifetime. There is an increased incidence of adverse effects not observed in young wild-type mice including altered coat condition, diarrhoea, eye abnormalities, abdominal distension, movement issues, tremors and seizures. A tiny minority of these develop tumours, but regular checking by our experienced animal technicians ensures these are detected early, and the mouse euthanised immediately. A specific code of practice for caring for aged mice is in place.

The adverse effects of irradiation can be infection or failure to repopulate the haematopoietic system. These effects, when they arise, are manifested within the first two

weeks following irradiation, most conspicuously as anaemia and weight loss. Some mice may lose pigmentation as a longer-term impact of irradiation.

The adverse effects of immunisation/infection or immunomodulation include systemic or specific tissue inflammation which will be transient, lasting for a few days. In the case of influenza virus there will be substantial weight loss which is restored within two weeks. Intermittent abnormal breathing may occur, but this will be transient lasting only a few days.

Some viruses will induce chronic infections with adverse effects such as weight loss and while this may be moderate in C57BL/6 other inbred strains manifest enhanced vascular permeability, lung immunopathology and animals will be closely monitored according to the humane endpoints detailed.

Tumour inoculation may lead to organ failure or affect breathing, feeding or drinking. Transplantation of human cells into non-irradiated NSG immunodeficient mice can lead to graft versus host disease (GvHD), but adverse effects will be reduced by introducing a genetically engineered Chimeric antigen receptor (CAR) into the transferred T cells. Cytokine release syndromes and neurotoxicity associated with these tumour immune models manifest as reduced activity, piloerection and weight loss.

The harmful manifestations of lymphoma may include reduced activity, intermittent hunching, marked piloerection, intermittent diarrhoea and occasionally intermittent abnormal breathing.

The adverse effects of GvHD include weight loss, which can become severe if not closely monitored. Signs of inflammation such as intermittent hunching, piloerection, flaking or reddening of the skin are also likely within days of the onset of GvHD.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for most of the mice will be mild or sub threshold Total animals used = 108070

- Mild: 24% (26440)
- Moderate: 2% (1830)
- Sub threshold: 74% (79800)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal experimentation is necessary because we want to understand the systemic properties of immune cells at the organismal level.

While we recognise limitations of mouse models and the need, where possible, to verify findings in other systems and species, we emphasise the mouse has proven overwhelmingly successful for the discovery of new cell types and the molecular genetic mechanisms underpinning their function. It has yielded essential basic knowledge of the immune system now being applied to improve the health of the humans and economically-important mammals.

Furthermore, it is extremely difficult to study lymphocytes in the bone marrow or lymphoid tissues of humans, because it is very difficult to obtain these from healthy donors.

Which non-animal alternatives did you consider for use in this project?

We have considered using cell culture methods, including organoids and do use these when appropriate. For example, we are using an in vitro model to investigate molecular pathways involved in germinal centre formation and plasma cell differentiation to complement the in vivo studies.

Why were they not suitable?

Features such as the distribution of lymphoid organs throughout the body and the intrinsic properties of lymphocyte recirculation cannot be recapitulated in tissue culture or organoids making investigations in the whole animal context essential.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals we will use based on our previous studies using these protocols. The numbers of mice required for the generation and rederivations of genetically altered mice are based on extensive experience of staff who regularly perform these protocols.

The use of colony management software and knowledge of the breeding performance of individual strains has enabled us to predict the numbers of mice of the correct genotype that we will produce from breeding, and the numbers of aged mice that we will need.

The numbers of mice required for experimental groups are based on power calculations, appreciation of variability and a knowledge of biologically meaningful effect sizes. We have factored in the need for experiments to be replicated independently and for greater variation in the responses of aged mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We reduce the numbers of animals used by paying careful attention to the design and planned analysis of the results including consultation when needed with a biological statistician in the Bioinformatics department and we use the NC3R's Experimental Design Assistant to ensure we are considering all relevant aspects of design. We perform our experiments in carefully controlled animal facilities that reduce biological and environmental variation; the use of optimised experimental procedures, including the use of genetically identical strains, blinding and randomisation to reduce technical variation; the multiparameter analysis of individual mice; the adaptation of new technologies such as the use of ribonucleoproteins to create knockouts or other genetic modifications; improving the sensitivity of techniques to enable measurement to be made on small cell numbers isolated from a single animal. For some experiments we are using in vitro methods to promote cell differentiation to defined states under controlled conditions, thus enabling access to cell numbers that would be unfeasible to obtain from mouse tissues.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where possible we will share mouse strains rather than generate new strains by transgenesis. Our mouse facility has an extremely efficient pipeline of tissue biopsies and rapid genotyping by a commercial provider, that provides timely results to enable efficient breeding.

Selective deletion of individual genes in specific tissues will be achieved by the use of Cre-Lox recombination to delete the gene of interest in defined cells. We employ rigorous quality control to ensure the fidelity of these systems. Pilot studies ensure minimum use of mice before decision points are reached in experimental design.

We have an established and highly successful programme of sharing of tissues with other investigators which we will continue.

We will prepare frozen stores of biological samples (tissue cells from infection and immunisation studies; bone-marrow; serum; and tissue sections) so that experiments can be performed on previously gathered tissues rather than using new mice.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use young-, adult- and aged-mice up to 26 months that are wild-type, transgenic, gene-targeted or contain reporter genes.

The small number of animals that will be embryo recipients, or undergo vasectomy, will experience transient pain due to surgery. This will be ameliorated by analgesia.

GA mice usually have a single gene altered, but multiple genes may be altered in the case of genetic redundancy or to combine gene mutation with reporters of cell fate. Typically, mutation is achieved by conditional deletion thus sparing any harmful or unwanted side effects of mutating a gene in all cell types of the body. In many cases, there is no pain, suffering, distress, or lasting harm expected with these models. In our facility which is pathogen free the animals with modified immune cells can live for normal lifespans without harmful effects of infection. The exception to this is for a small number of animals with abnormal immune regulation that may experience some symptoms of autoimmune or inflammatory disease - where mice displaying clinical signs will be killed and used for tissue collection before clinical signs exceed moderate severity.

Some animals will experience infections or immune challenges that will elicit immune responses. These experiments will be further refined by using Cas9-based methods to inactivate genes in cultured primary lymphocytes. This will refine some experiments in which cre-expression may cause unwanted effects that are difficult to control for. It will also reduce the numbers of animals bred.

The use of non-replicating antigens, vaccines, and attenuated microorganisms and in some cases vaccine strains (which have limited pathogenicity) will enable questions about immune responses to be answered.

Some experiments will use protein degron technology (where short amino acid sequences - as degradation signals - are used to manipulate protein degradation) as an alternative to genetic deletion of the gene. This important innovation allows protein ablation, but unlike gene ablation it is revertible. Moreover, it is not limited by the features of the cre-lox system which requires Cre to be expressed at a specific time.

Some experiments will involve injecting sources of haematopoietic cells to reconstitute the immune system. These experiments provide detailed in vivo knowledge that studying cells ex vivo cannot do. This can occasionally lead to ill health, either as a consequence of the irradiation or graft versus host disease, but these mice will be carefully monitored and any animal exhibiting moderate pain or distress will be culled.

We will use a specific type of immunodeficient mice (NSG) for transplantation of human tumour cells as these are considered to be a relevant preclinical model for adoptive cell therapy.

Suffering will be minimised by provision of analgesia where appropriate, provision of diet-gel food on cage floors should animals have difficulty accessing water due to mobility impairment, sub-cutaneous hydration if dehydration is apparent, housing in heat room/on heat pad if temperature drops significantly (e.g., following anaesthesia).

Why can’t you use animals that are less sentient?

Importantly, 'less sentient' species are not applicable for studies of B and T cells and the choice of species is limited by the fact that in evolutionary terms the adaptive immune system is largely a vertebrate invention. Even so, in some vertebrate species such as Zebrafish, the properties of lymphocytes differ significantly from humans and mice making them interesting but unsuitable for our project.

The mouse is the species of choice because of the extensively validated tools and resources available for quantitative immunological phenotyping and mechanistic studies across the life-course. Studies of ageing in the mouse have provided key insights into human immune systems but require the long-term care of mice with which we have much experience. The availability of inbred stains and well annotated genomes is important for our studies as they develop into the investigation of molecular mechanisms ex vivo and in vitro. The requires careful and attentive monitoring of breeding programmes and quality control.

Early-stage mouse embryos are unsuited to these studies as the adaptive immune system and immunological memory is a feature of adult animals and we propose to study these features in adulthood and ageing animals.

Terminal anaesthesia is inappropriate for long-term experiments that require systemic immune responses, but may be used to prepare tissues for further study.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are mindful of the increased incidence of poor health for some individual mice as they age and that this may be affected by genetic modification, which may have a positive or negative impact on this process. We observe these mice with increased frequency and have developed a detailed checklist of possible age-related changes, and procedures for

monitoring and treatment. Data gathered from these studies will be available to further refine best practice.

We will implement any refinements developed by our animal house staff, who have a long history of innovative practice, including environmental enrichment.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All experiments which will integrate refinements from the NC3Rs such as the ARRIVE guidelines on design and reporting; the LASA aseptic guidelines; LASA Diehl guidelines on volumes and frequency limits and the most up-to-date veterinary knowledge.

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How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about advances in the 3Rs during this project through attention to the work and outputs of the NC3Rs including workshops and webinars; by invited seminars on the 3Rs; and seminars on Research Integrity - which covers experimental design and data management. We will actively stay updated with our field of research through collaboration, conference attendance and reading the literature which frequently highlights innovations (e.g., Cas9 methods). We will use Home office advice made available to us through our dedicated Home Office Liaison.

To implement advances effectively, we will follow guidance from our local AWERB. Our animal facility also has a dedicated Strategy Committee and a User group, into which we influence and are made aware of emerging best practice. These groups discuss and make collective decisions about advances in the 3Rs and advise on how they can be implemented, both across the organisation, and by individual researchers.

87. Molecular Genetics of Hearing and Deafness

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Hearing, Deafness, Genetics, Therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify and characterise genes required for mammalian hearing, and to elaborate upon the mechanisms of hearing loss. Provide proof-of-principle intervention studies to test the potential of possible therapies to restore or preserve hearing function.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Hearing impairment is the most common sensory deficit in the human population. According to figures from the World Health Organisation there are currently around 360 million people worldwide living with mild to profound hearing loss, which includes all types of hearing loss (e.g. conductive and sensorineural) and age of onset (e.g. congenital, early-, and late-onset). Approximately half of these cases have a genetic basis, with the hearing loss occurring either as an isolated condition (non- syndromic, 70%) or presenting with additional phenotypes (syndromic, 30%). Around 150 non- syndromic loci have been identified in human and over 400 genetic syndromes are known that include deafness. To date, around two-thirds of the genes for non-syndromic deafness loci are known, and while the causative genes for several of the most common deafness syndromes have been elucidated, the vast majority of genes underlying syndromes with deafness are unknown. As such, we are far from having a complete understanding of the genetics underlying hearing function. In addition, a recent Lancet Commission on Dementia identifies midlife peripheral hearing loss as the highest individual risk factor for dementia, and this is pertinent as age-related hearing loss (ARHL) is the most prevalent sensory deficit within the population affecting >30% of individuals older than 55-years. However, currently little is known about the genes underlying ARHL.

Mouse genetics has played an important role in our understanding of the development and functioning of the mammalian auditory system. Utilising forward-genetic screens many deafness mouse mutants have been identified, and characterisation of these have enabled genes critical for hearing function to be elucidated. Allied with gene-driven approaches, mouse mutants continue to help elaborate the molecular mechanisms and physiological bases of human hearing impairment. Furthermore, mouse models offer the opportunity to study cochlear pathologies resulting from mutation of these ‘hearing’ genes, something that is not possible in humans due to the cochlea being encased within the hardest bone in the human body. Moreover, the increased understanding of the biology of hearing disease gained from studying mouse models will continue to allow the advent of therapeutic interventions designed to protect or restore hearing, and potentially reduce dementia incidence.

What outputs do you think you will see at the end of this project?

The identification and characterisation of new mouse models of hereditary hearing loss will increase our understanding and knowledge of how the mammalian auditory systems functions. Moreover, by generating mouse models carrying human deafness-causing mutations we will be able to identify the pathological changes occurring within the cochlea, which is important for understanding disease onset and progression. Investigation of these models will help to further define the molecular and cellular basis of hearing and hearing loss.

Over the next 5-years, it is our intention to: validate and characterise up to 6 candidate deafness genes by generating and phenotyping the appropriate mouse model; elaborate upon the functional interactome of known 'hearing' genes; and, identify up to 3 therapeutic targets for future work.

The primary objectives of this project are to:

1. To breed and maintain genetically altered mouse models

2. Identify and functionally characterise novel genes required for mammalian hearing.

3. Establish new insight to the molecular mechanisms and pathological processes involved with hearing loss.

4. Undertake proof-of-principle studies of potential therapies for auditory dysfunction.

Each novel model that we characterise has the potential to implicate additional molecular targets for investigation, which we will explore in partnership with collaborators from academia and industry.

Moreover, through the validation of human loci and genetic variants we have the potential to provide a genetic diagnoses for patients with idiopathic hearing loss.

Integration of our new data with established knowledge will be facilitated through publication and presentation of our findings.

Who or what will benefit from these outputs, and how?

Our research will have impact in the short-term, and in the long-term after the completion of the project.

In the short-term, the data and resources (mouse models) produced as part of this project will be of great interest to UK and international researchers. We will produce data that will determine the role of specific genes in the function of the mammalian auditory system, and identify the molecular and cellular consequences associated with perturbation of these genes. These new data will be fed back directly to clinicians and patient groups, including large-scale human projects such as the Genomics England 100K Genomes programme, to help provide patients with a genetic diagnosis.

A key long-term benefit will be the provision of new genetic pathways and fully-characterised mouse models that can be tested to determine if they can be modified by therapies to delay or prevent hearing loss. These models will be made available to the scientific community. Moreover, our findings will continue to be disseminated through publication in open-access, peer-reviewed journals and presentations at scientific conferences.

How will you look to maximise the outputs of this work?

It is common for sensorineural hearing loss to be part of a syndromic disorder e.g. Usher (deaf- blindness) syndrome, and many metabolic and neurological disorders. In particular, there is a growing literature suggesting age-related hearing loss is causally associated with cognitive decline and having an increased risk of dementia diagnosis. While we are able to assess our hearing loss models for some of the classic symptoms pertaining to these additional disease areas (e.g. vision, metabolism, neurobehaviour and neurodegeneration), we always look to work with collaborators who are expert in their respective fields to ensure our models are fully characterised.

Dissemination of the new knowledge generated by these works will involve presentation of findings at major international scientific meetings and publications in open-access, peer-reviewed journals. This will include communicating our unsuccessful approaches as well as our successes.

Species and numbers of animals expected to be used

- Mice: 27400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

While it is possible to perform genetic manipulations in flies, frogs and fish, the processes being studied here are being studied in a mammalian context and although other animal and non-animal species can be informative in this regard, they cannot supplant studies specifically in mammals. While flies and fish both have mechano-sensitive cells that are required for stimulus detection, and can be thought of as similar to the sensory cells required for mammalian sound reception, it is only mammals that have evolved cochleae - the organ of mammalian hearing. As such, mice remain the model of choice for studying the mammalian auditory system due to: the similarities in structure and function of the inner ear; the wealth of the genomics information available; their relevance to humans; and, the technologies to be able to generate, establish, cryopreserve and disseminate mouse colonies.

Moreover, well-developed and characterised inbred strains of mice present an opportunity for reducing variability (thus enhancing reproducibility), and for identifying genetic interaction between two or more genes.

Hearing loss is a common birth defect and it is also the most prevalent sensory deficit experienced by the elderly, as such we are interested in both early- and late-onset hearing loss, and to do this we will study neonatal, juvenile, adult and aged mice. Importantly, the mouse offers the opportunity to study the progressive (age-related) changes associated with the onset and progression of hearing loss, something that is not possible in humans.

In addition to altering genes, we are interested to understand how environmental factors such as noise impacts upon the onset and progression of hearing loss.

Due to the unknown nature of the GA lines that will be tested it is possible that some may not be viable, in these circumstances we may study embryos to help identify why the mice do not survive when a particular gene(s) is perturbed.

Typically, what will be done to an animal used in your project?

Many of the mice on this licence will be used only for breeding. This is due to the complex nature of hearing and the panoply of genes implicated in hearing loss, with our aim being to understand these genes and the cells that affect this disorder. This sometimes requires several steps of breeding to obtain a group of mice with the necessary combination of genes that can be studied. We will also maintain breeding lines to ship to collaborators (authorized to accept GA lines) to allow specialised studies to be undertaken, with the aim of maximising our understanding of the hearing deficit in our mice while also reducing overall animal use.

Approximately 4,000 mice will undergo phenotype testing to characterise preclinical models and for hypothesis-testing. The majority of mice will undergo single, or recurrent, auditory threshold testing, which involves anaesthesia used for immobilisation. Some will experience a combination of tests over their life-span, most of which are non-invasive, although a small number involve anaesthesia used for immobilisation, for imaging, physiology recordings, or the administration of substances to modulate gene expression.

Typically, mice undergo a final terminal study, performed under anaesthesia, and in some cases a terminal bleed or perfusion may be carried out. All experiments are expected to end by 78 weeks of age.

What are the expected impacts and/or adverse effects for the animals during your project?

There are many possible adverse effects that could occur due to genetic modifications of the mouse genome. Moreover, in some cases we will be breeding the genetic alteration to homozygosity for the first time. Possible adverse phenotypes are those that lead to sub-viability such that homozygotes die between birth and weaning.

The genetic alterations studied in this project may lead to hearing loss in the mice, which in itself does not lead to overt adverse effects in mice. However, due to the developmental, physiological and genetic similarities between the cochlea (organ of hearing) and the vestibular apparatus (organ of balance) that together comprise the inner ear, there is the potential for deafness genes to also cause vestibular dysfunction leading to balance defects. In severe cases this may manifest as a stereotypic circling behaviour.

Moreover, it is common for sensorineural hearing loss to be part of a syndromic disease e.g. Usher (deaf-blindness) syndrome and many metabolic and neurological disorders. As

such, other adverse effects that might occur during the lifetime of the mice include deterioration in health, tremors and lack of condition.

Also, there is a growing literature suggesting age-related hearing loss is causally associated with cognitive decline and having an increased risk of dementia diagnosis. As such, genetic alterations in the mice used in this project may lead to the development of dementia-relevant phenotypes. In mice these changes may lead to: increased aggression/fighting; decreased food intake leading to weight loss; or, changes in memory and behaviour that may not be apparent in the home-cage, but require additional testing to measure.

For auditory threshold testing mice will need to undergo anaesthesia. All anaesthetic use in mice carries a risk of mortality, which may differ between genetically altered lines.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

14,000 mice on the mild breeding and maintenance protocol are not expected to suffer any adverse effects and the vast majority will not reach higher than a sub-threshold severity.

On the moderate breeding and maintenance protocol, it is anticipated that any mice carrying the disease-causing phenotype could exhibit a moderate phenotype. Moreover, it is anticipated that of the GA lines being bred to homozygosity for the first time, 50% could exhibit a moderate phenotype. As such, approximately 25% of the 2,100 mice may suffer a moderate severity.

4,000 mice on the phenotype characterisation protocol 90% expected to reach a moderate severity. This is in part due to the phenotype of the mice, in which the genetic alteration could lead to a moderate severity. However, this mostly reflects that the majority of these mice will undergo a hearing test and the combinatorial effect of repeating mild tests over the life span of the animal in order to understand the progression of disease. This will lead to an overall moderate experience for aged animals that have undergone recurrent longitudinal phenotyping.

Of the 500 mice expected to experience a noise-challenge, 100% will reach a moderate severity due to all receiving at least one hearing test.

Of the 500 mice to be used to assess therapeutic interventions for hearing loss, 100% will reach a moderate severity due to all receiving at least one hearing test.

What will happen to animals at the end of this project?

- Killed

- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The mammalian auditory system is highly complex, having a sophisticated, tonotopic organisation that needs to be maintained from the cochlea to the brain. Given the complexity of the auditory apparatus, with its myriad of interacting cell types, it is impossible at this juncture to reproduce and study this biological system using non-animal alternatives. In particular, the combined use of genetics technologies allied to the study of the cochlea during the ageing process, or in response to noise, would not be possible in an in vitro or ex vivo system.

Mice continue to be the predominant model organism for hearing research. Similarities in auditory structure and physiology between mice and humans, the close evolutionary relationship of genomes, short lifespan, genetic standardisation and the available genetic toolkit together make the mouse a useful and crucial model organism for studying the functional genomics of the mammalian auditory system. Furthermore, we are interested in identifying co-morbidities in other organ systems (e.g. syndromic hearing loss) and hence a whole animal approach is required.

Which non-animal alternatives did you consider for use in this project?

As we begin to functionally characterise our new mouse models of hearing loss and to investigate the underlying molecular mechanisms we may be able to make use of newly developing techniques. For example, Liu et al (2016) have reported a 3D culture system that can generate large numbers of functional sensory cells. However, these ESC-derived organoid hair cells resemble vestibular hair cells rather than auditory cochlear hair cells. More recently, McLean et al (2017) have shown they are able to take acutely dissected organs of Corti and, in a 3D culture employing a cocktail of drugs and growth factors, clonally expand these cells into a nearly pure population of hair cells in high yield. These could be an alternative if wanting to study sensory hair cells in isolation (i.e. mechano-transduction).

However, our work is focused on trying to elaborate upon the mechanisms involved in the maintenance of cochlear function, and trying to understand the genetics of progressive and age-related hearing loss. Unfortunately, the current state-of-the-art inner ear organoids do not permit these types of studies as they model immature hair cells in isolation.

While non-mammalian model organisms (e.g. fly, worm and fish) are unsuitable for many auditory studies they can be employed for preliminary expression analyses to help prioritise genes for further investigation. Indeed, we continue to work with collaborators in the USA to assess the expression of novel hearing loss genes arising from the International Mouse Phenotyping Consortium (IMPC) programme in the fly.

Over the duration of this licence we will continue to monitor the development of new in vitro and ex vivo systems, and if suitable ones become available we will replace the relevant in vivo assay.

Why were they not suitable?

Currently, we cannot grow or replicate the cochlea in a dish. While there is much interest in trying to develop organoids for auditory research, at present using this type of approach we are not able to recapitulate: the complexity of the mammalian auditory system; ageing within a body; or, the response to noise exposure. Moreover, using in vitro or ex vivo cell-based studies alone we cannot identify the wider ranging effects of a gene across multiple body systems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

A recent study utilizing the UK Biobank resource has identified 44 independent associated genomic loci for self-reported adult hearing difficulty. We have been generating knockout (KO) mouse models for these loci/genes, and have been recurrently phenotyping cohorts of mice to 18-months of age. If a hearing deficit is identified, the KO model is then further characterized to determine the associated pathobiology and elucidate the molecular mechanism leading to the hearing loss. Going forward, we intend to continue to elaborate upon the genetics of human ARHL through the validation of additional genes showing association with adult hearing difficulty. In the first instance, KO models for these genes will undergo validation studies, if these are found to have an auditory phenotype the model will require further characterisation. In addition, the spatial and temporal requirement of these genes may be further investigated using a conditional KO approach. Provided here are example breeding schemes that will be employed:

To validate candidate hearing loss genes of interest, a cohort breed will be undertaken using the cross GeneX+/- x GeneX+/-. To obtain the three required genotypes GeneX+/+, GeneX+/-, and GeneX-/-. This will be undertaken on the C57BL/6N-repaired background.

16 mice (8 females and 8 males) per genotype group will be used. Therefore, 48 mice (24 males and 24 females) will undergo longitudinal auditory and behavioural phenotyping, as well as terminal tissues being taken for histopathology and imaging studies. For each gene, to obtain 8 mice per sex genotype will require 12 litters, producing ~80 pups.

To characterise a confirmed new hearing loss gene, breeds will be undertaken using the cross GeneX+/- x GeneX+/-, to obtain cohorts containing the three required genotypes GeneX+/+, GeneX+/-, and GeneX-/-. These cohorts will each be aged to specific time points to allow progressive pathological changes occurring within the cochlear to be determined. From our experience of similar previous studies, it is likely that 8 mice (4 females and 4 males) per genotype group will be sufficient to identify histological changes. Therefore, 24 mice (12 males and 12 females) will be aged for terminal tissues to be taken for histopathology and imaging studies. For each aged cohort, 4 mice per sex genotype will require 7 litters, producing ~50 pups. It is expected that four timepoints will be investigated.

To ascertain the spatial and temporal requirement of hearing loss genes, conditional knockout mice will be generated utilising a ‘hair cell-specific’ (Myo15-cre) and a ‘global inducible’ (Ubiq-creERT2) cre driver line, respectively. Using the Myo15-cre as an exemplar, the cohort breed will be undertaken using the cross: GeneZfl/+; Myo15cre/+ x GeneZfl/+; Myo15+/+. To obtain the ‘Test’ animals (GeneZfl/fl; Myo15cre/+), and the five required ‘Control’ genotype groups (GeneZfl/+; Myo15cre/+, GeneZfl/+; Myo15+/+, GeneZ+/+; Myo15cre/+, GeneZ+/+; Myo15+/+, GeneZfl/fl; Myo15+/+). On a C57BL/6

background. For each cohort breed, 8 mice (x4 females and x4 males) per genotype group (x6) will be used. As such, for each cohort breed 48 mice (24 males and 24 females) will undergo longitudinal auditory and behavioural phenotyping. To obtain 4 mice per sex genotype will require 13 litters, producing ~80 pups.

To investigate potential therapeutic interventions for hearing loss, biological substances will be delivered either directly to the inner ear, or systemically, to wild-type and homozygous mutant mice. A typical experiment will involve performing 'experimental' and 'control/sham' injections on a cohort of mice (10 mice/genotype/sex/intervention). As such, for each cohort breed 80 mice (40 males and 40 females) will enter an intervention study. To obtain 20 homozygous mice per sex genotype will require 29 litters, producing ~200 pups.

To generate the required experimental cohorts and to support the maintenance of the parental lines (including cre-driver lines etc), in total we estimate we will need to breed around ~22,500 mice for this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Prior to embarking on a project the absolute number of mice required for the phenotyping assay will be determined. We will employ best practice in experimental planning, including using statistical approaches such as power and resource equations based on real data sets, to predict the number of animals required.

Where possible, we will maximise the data obtained from each cohort of mice. Combining tests in the same mice allows the interpretation of data to be correlated directly, rather than inferred. This allows us to carry out more advanced statistical analysis to detect correlated trends between assays. Directly correlated effects have a greater sensitivity as the major source of variance (between mice) is removed, enabling the detection of subtle phenotypes. We will also phenotype over the life-span of the animal to improve power and to understand the progression of disease and effect of age.

Standard Operating Procedures have been written, which standardise the way data are collected. Thus, reducing variability and consequently the required sample size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Most crosses use dynamic mating systems, where the male is removed prior to littering thereby avoiding second litters and the risk of overbreeding.

Mouse lines will only be maintained while there is a justified use for their continued breeding and will be cryopreserved for future collaborations or the use of other scientific groups. All breeding schedules will be informed by the fecundity and productivity statistics calculated from previous experience of breeding any animal models used.

Whenever possible, genetically modified lines will be sourced from repositories to avoid re-making of lines.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Hearing loss in itself is not painful. However, in a syndromic form it can be associated with other, more debilitating, conditions. We are particularly interested to elaborate upon the genetics and pathobiology of age-related hearing loss, which while generally termed non-

syndromic, has been reported to be a risk factor for dementia diagnosis as well as being associated with depression, social isolation and frailty.

To date, little is known about the genetics of age-related hearing loss and how this condition might lead to dementia. This project aims to identify robust models for progressive hearing loss and to investigate these for signs of cognitive decline and other co-morbidities. These models will not only provide an understanding of the molecular causes underlying auditory decline, but will offer an opportunity to test interventions to prevent or slow age-related hearing loss.

Test

Why is this the most refined method

Ear biopsy

Mice need to be ear clipped for identification and the same piece of tissue is used for genotyping. Genotyping protocols have been optimised to use these very small samples to avoid the need for other samples to be taken. This is more refined than other methods such as a tail biopsy.

Induction of transgene expression and changes in gene expression using injection of genes

In most cases this will be done by oral dosing, which is more refined than injection, as while it involves restraint there should be no pain. Injections may be necessary depending on the type of recombinase line used and the age at which it needs to be activated. These methods can be used to avoid developmental issues that may prevent a mouse from growing properly, which may therefore reduce the number of mice needed for some experiments. We will continue to review the protocols used for transgene induction, and will modify these to current best practice.

SHIRPA

SHIRPA is an observational test that involves placing the mouse in an arena or a jar and looking for abnormalities. This is non-invasive and can provide a lot of phenotypic information that can be followed up in further, more complex, tests. This is also a good opportunity to identify subtle welfare issues.

Click box

This test is non-invasive and involves monitoring for the movement of the ear in response to a brief high-frequency loud sound stimulus. This provides an opportunity to assess the hearing of a mouse before going on to more in-depth ABR studies, and can inform upon the utility of other phenotyping tests that rely upon a reaction to a sound stimulus.

Swim test

This is a non-invasive test that involves placing a mouse in a tank of warmed water and monitoring their ability to swim normally. Irregular swimming, immobile floating or tumbling are all indicative of abnormal vestibular dysfunction.

Open field

Open field provides data on the reaction of a mouse to a novel environment. The arena is anxiety-inducing in that the mouse has not seen it before and the light is relatively bright. There are no smells or sounds that could cause stress to the animal.

Rotarod

Rotarod is useful to measure activity when a mouse is encouraged to run by placement on a moving rod. It can also be used for cross-laboratory bench-marking to ensure reproducibility of phenotype.

Acoustic startle and PPI

For this test it is necessary to assess the response of the mouse to an audible stimulus. While the stimulus itself is anxiety-inducing, efforts are made to reduce stress by having a constant background noise and performing the test in the dark. A protocol has been developed over many years to maximise the capture of useful data in a short time period.

Ophthalmoscope

For this test the mouse is held while the eyes are examined. This is more refined than more in-depth imaging as it does not involve general anaesthesia. However, the data collected are more subjective and there may be times when in-depth imaging is needed, in which case mice will undergo optical coherence tomography (imaging the eye under general anaesthesia).

Optokinetic drum

This test involves the mouse being placed on a platform in a large enclosed environment under low-lighting levels. The mouse is completely surrounded by monitors which display black and white lines of various widths and moving at various speeds. This assesses the visual response to movement stimuli and adds to the phenotypic information gathered in other eye tests.

Pupillometry

This test measures the pupillary response to light to measure the non-visual functions of the eye. This test is non-invasive, but does require the adjustment to a dark environment and the exposure to a bright light.

Behavioural observations

This test is a non-invasive observational test whereby mice are placed either in an arena or are observed in their home-cage. This can provide a lot of phenotypic information that can be followed up in further, more complex, behavioural tests.

Grip strength

The grip strength test is the quickest, least invasive way of measuring muscle strength. The test lasts for less than one minute and should cause no pain, only stress induced by handling.

Gait analysis

This involves placing the mouse in an arena and video-recording for a short time. This is the most refined method of running test as, unlike some other gait analysis equipment, it does not involve any stimulation to force the mouse to move.

Hanging wire test

This non-invasive test involves placing a mouse on a wire grid (e.g. cage top), which is then inverted and the latency to fall is recorded. This test evaluates motor neuromuscular function.

Spontaneous alternation/Y-maze

This is non-invasive and involves placing a mouse in an arena and allowing it to explore. Light levels are set so that the mouse can see, but are not intended to be alternation/Y-maze anxiety-inducing. Spontaneous alternation provides a measure of spatial working memory, as mice should display a tendency to explore new areas.

Elevated plus/zero maze

This is non-invasive and involves placing a mouse in an arena and allowing it to explore. Light levels are anxiety-inducing in some areas of the arena, but are darker and not anxiety-inducing in other areas. This is necessary as the test assesses the preference of the mouse for the darker or lighter areas.

Marble burying test

This non-invasive test involves placing a mouse in a cage containing marbles Marble burying test arrange in an ordered pattern. After a set period of time, the animal is removed and the number of 'buried' marbles is counted. This test is used to assess repetitive and anxiety-related behaviour.

Social recognition test

Light levels are low to reduce anxiety. In this test another mouse is present in a section of the arena. This mouse is in a small cage to stop any aggression, but the scent and sight of it are needed to understand how the mouse behaves to a social stimulus.

Barnes maze

This is non-invasive, spatial learning and memory test that involves placing a mouse in an arena and allowing it to explore. Light levels are set so that the mouse can see, but are not intended to be anxiety-inducing.

Recording vocalisations

This is a non-invasive test that involves placing a mouse in a modified 'home-cage' to allow the subsequent recording of vocalisations under normal housing conditions. Ultrasonic vocalisations can be employed as a behavioural marker of disease progression in certain neurodegenerative diseases.

Locotronic analysis

This non-invasive test involves placing a mouse on a horizontal ladder and, as the animal walks along the ladder, foot misplacement is measured. This is a measure of sensory/motor function.

Light dark box

This is non-invasive and involves placing a mouse in an arena and allowing it to explore. Light levels are anxiety-inducing in some parts of the arena, but not others. This is necessary as the test assesses the preference of the mouse to the darker or lighter areas.

Echo-MRI

This test involves less than one minute of light restraint whilst body composition is measured. This is more refined than alternative tests that require general anaesthesia and a longer time to gather the data.

Urine collection

Mice are held and urine caught in a container, or in some cases mice are placed on a plate or a modified cage with specialist substrate (i.e. treated sand) that allows for urine collection. In both cases this is less stressful than placing the mouse in a metabolic cage and collecting urine overnight.

Home-cage monitoring

This is non-invasive and measured in the home-cage. After the initial insertion of a microchip under a light sedation, this test involves no further pain, suffering or distress to the mouse.

Olfaction testing

This is a non-invasive test that can be undertaken in the home-cage or an arena. These behavioural experiments allow assessment of olfactory function, including olfactory habituation, discrimination, odour preference/detection/sensitivity, and olfactory memory, to either social or non-social odours.

Electroretinographyof (ERG)

This test is done under general anaesthesia due to the extremely sensitive nature the measuring electrodes and the need for contact lenses to be held over the eyes of the mouse. This test measures the electrical responses of the eye to light stimuli. The electrodes are fine enough to not cause pain.

Auditory brainstem response (ABR)

This test is done under general anaesthesia due to the extremely sensitive nature of the measuring electrodes and the need for a sound-attenuation chamber. Mice are monitored at all times through a window and the electrodes themselves cause no pain. This test measures the electrical activity in the brainstem in response to sound stimuli.

Distortion product otoacoustic

Otoacoustic emissions are responses generated by the sensory outer hair cells (OHCs) when the cochlea is stimulated by two pure tone frequencies. This test,emission (DPOAE) performed under terminal anaesthesia, is used to determine the status of thecochlea in vivo.

Endocochlear potential (EP)

The EP is a positive voltage of 80-100mV observed in the endolymphatic fluid within the scala media compared with the perilymphatic fluid found in the remainder of the cochlear duct. The generation and maintenance of the EP is essential for the proper function of the cochlea, it is measured under terminal anaesthesia using an electrode advanced directly into the endolymph.

Blood sampling

Blood samples are collected to a maximum of 15% total blood volume of the animal, which has shown no adverse effects in previous studies. Samples are taken from the tail vein using a very small cut and mice have a local anaesthetic applied to the area twenty minutes before.

Why can’t you use animals that are less sentient?

The mouse continues to be the predominant model organism for hearing research, in part due to the close similarity in the structure and physiology of their auditory apparatus when

compared with that of the human ear. Additional strengths of the mouse as a model for studying the functional genomics of the mammalian auditory system include: the close evolutionary relationship of their genome to humans; genetic standardisation; and, the genetic toolkit available for the manipulation of their genome. Moreover, the complexity of hearing means that simpler model systems, such as fish and drosophila, do not allow for all aspects of the mammalian auditory system to be studied. In particular, fish and fly do not possess a cochlea, the mammalian organ of hearing.

Furthermore, we are interested to elaborate upon the genetics and pathobiology of age-related hearing loss, which is something that cannot be fully explored in flies or fish due to differences in the anatomy, structure and function of their auditory apparatuses. Moreover, investigating a progressive disorder, such as age-related hearing loss, requires longitudinal ABR phenotyping to monitor and assess auditory decline with advancing age, which requires multiple general anaesthetics with recovery.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For all tests it is important that the animal has no additional stress, therefore mice are handled calmly and habituated to testing rooms as well as arenas if possible.

For all tests mice are only housed in modified cages or arenas for the minimum time needed to gather meaningful data. Mice undergoing phenotyping tests are monitored more frequently and are removed from tests if they appear to be suffering from an adverse stress reaction, or other unexpected adverse effects of the phenotyping tests.

Mice which have had anaesthesia have extra monitoring until fully recovered and extra checks when back in the holding rooms. When general anaesthetics are necessary, the combinations with least adverse effects will be used, for example for all tests inhalation anaesthetics will be used, with the exceptions of Auditory Brainstem Response, Electroretinography and Optical Coherence Tomography, which cannot be carried out on a mouse with a face mask. Pain from tail bleeds is reduced by using local anaesthesia.

All surgery will be undertaken in full compliance with Laboratory Animal Science Association aseptic technique guidance to minimise infection risk.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Routes and volumes for administration of substances are taken from Laboratory Animal Science Association good practice guidelines: administration of substances 1998 (http://www.procedureswithcare.org.uk/lasa_administration.pdf).

Surgery will be undertaken as per the Laboratory Animal Science Association Guiding Principles for Preparing for and Undertaking Aseptic Surgery 2017 (http://www.lasa.co.uk/wp- content/uploads/2017/04/Aseptic-surgery-final.pdf)

The animal house has full AAALAC and ISO9001-2015 accreditation. To conform to these standards we must ensure a high level of quality control on all fronts including husbandry, phenotyping and administrative processes.

Standard operation procedures for most tests have been generated using data and expertise from multiple animal houses and can be found at https://www.mousephenotype.org/impress

ARRIVE and PREPARE guidelines will be followed at all times.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Over the course of the project I will attend general 3Rs symposia, read the NC3R and RSPCA newsletters, and receive regular email updates from the Animals in Science Committee Secretariat. Also, as Chair of an Animal Welfare and Ethical Review Body (AWERB) I am in a position to hear about new opportunities for refinement. I will also attend auditory-focussed conferences to learn about new opportunities for 3Rs specific to my area of scientific research.

88. Genes and Environment in Diabetes

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

diabetes, genetics, microbiome, prevention, immunity

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Diabetes mellitus is caused by inherited (genetic) factors and non-inherited (environmental) factors. The aim of this project is to understand the impact of genetic variations and environmental conditions on the development of diabetes mellitus, in order to design better approaches to disease prevention and treatment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This work is important because, according to the World Health Organisation, there are more than 400 million people throughout the world living with diabetes and it leads to 1.5 million deaths annually. This work will help us to understand the genes and mechanisms that are important in diabetes risk, and also whether there are any environmental factors (e.g. use of antibiotics) that make diabetes more or less likely. This information is critical to preventing diabetes in future.

What outputs do you think you will see at the end of this project?

The outputs of this project will include information about which genes have the strongest effect on diabetes risk and how they function in the immune system and in the development of diabetes.

The project will also provide information on environmental factors that may influence the immune system and the onset of diabetes such as the use of antibiotics, or particular dietary components. Environmental factors under investigation include the gut microbiome, which is a term used to describe all the micro-organisms, such as bacteria, living in the digestive tract.

The data that will be shared with other researchers will include scientific publications describing the findings of the work, as well as detailed datasets being deposited in online resources for scientists to use and analyse.

Who or what will benefit from these outputs, and how?

The short-term outcomes of this work (during the lifetime of the project) will be an improved understanding of the function of particular genes, especially with respect to the immune system and the development of diabetes.

Understanding which genes make the greatest contribution to diabetes risk, and how they do this, will help to identify new treatment or preventative targets in diabetes. This information will begin to emerge within 3-5 years, and in the longer term (5-10 years) would potentially translate to a clinical benefit to patients.

How will you look to maximise the outputs of this work?

Data generated by this study will be disseminated by conference presentations and the publication of scientific papers.

We will continue to network and collaborate with colleagues locally, nationally and beyond to share best practice and ensure that work is not duplicated.

We will also ensure that were benefits are identified with more general applicability, outside the field of diabetes, we will communicate these to the relevant audience.

Species and numbers of animals expected to be used

- Mice: 12000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mice for this research as there are well-characterised spontaneous models of diabetes mellitus in this species that can be used to study the impact of genetic mutations in the development of disease. Some genes that are potentially important in diabetes are conserved across a range of species including mouse, rat, dog, zebrafish and chicken, but many genes of interest are not found in C.elegans (worms) or Drosophila (fruit flies). It is challenging to undertake diabetes-related research on zebra fish, as unlike mice - where a simple urine test is possible, it is impossible to make a diagnosis of diabetes non-invasively in a fish.

Typically, what will be done to an animal used in your project?

Typically we will use a strain of mouse that has a high possibility of developing spontaneous diabetes mellitus from the age of 11 weeks, meaning that from this age the mouse will undergo regular (typically weekly) urine tests for to detect diabetes as early as possible.

In some cases, the mice might have a mutation in a particular gene of interest that will allow us to study the impact of this change on the immune system and on the development of diabetes.

We might also test the immune system further by administering a substance designed to stimulate the immune system and taking blood samples to measure the response.

In some mice, we will try to change the composition of the microbes (for example bacteria) in the gut, for example using antibiotics, to see if this changes the risk of diabetes. The immune system of these mice might also be monitored by blood sampling.

What are the expected impacts and/or adverse effects for the animals during your project?

If diabetes develops, this can cause excessive thirst, passing of large volumes of urine, and weight loss. If not recognised early, it can progress within days to weeks to cause reduced movement, quiet behaviour and reduced appetite.

Administration of substances to challenge the immune system should not be associated with anything other than transient pain (if the substance is injected). Animals are monitored

closely for unexpected responses to challenge of the immune system, but with the substances and doses planned, adverse effects are not expected.

Alteration of the microbes (for example bacteria) in the gut (e.g. by administration of antibiotics) is not expected to lead to adverse effects, although occasionally transient diarrhoea can be seen.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- 50% Sub-threshold
- 40% Mild
- 10% Moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The immune system and the development of diabetes is very complex. Diabetes itself is the result of many genetic variations, combined with a wide range of environmental triggers in a whole organism. It is a complex disease affecting the whole animal and so cannot be replicated in a test tube. Other organisms such as fish and worms do not have diabetes, nor do they have the genes being studied, so it is not possible to do these studies on these species.

We work on the human immune system and collaborate with other laboratories studying the human immune system, so wherever possible we will translate findings to human studies as early as is feasible.

Which non-animal alternatives did you consider for use in this project?

We considered (and actively use) tissue culture for examining the impact of genetic mutations on cell function, and this informs the work we undertake with animals. However in a cell culture model it is impossible to replicate the complexity of the pancreas, and to measure diabetes as a potential outcome of genetic variation.

We also, wherever possible, consider findings from human studies before undertaking studies in mice.

Why were they not suitable?

Whilst we can learn some information by mutating a gene within a cell line in tissue culture, in a cell culture model it is impossible to replicate the complexity of the pancreas, and to measure diabetes as a potential outcome.

In human studies, genetic variations typically have a very small but real effect on diabetes risk, and environmental conditions are highly variable, therefore it can be extremely challenging to learn about the function of genes that have been associated with diabetes risk.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Several factors have gone into this calculation:

1. This will be the third licence we will have held for diabetes research, therefore we have been able to make estimates based on results from previous years

2. We have also considered mouse numbers within the context of the expected funding and staffing available for the work.

3. We know from experience that non-obese diabetic (NOD) mice can have very large litters (up to 15 pups) so this has also been considered in our calculation.

4. We will use the minimum number of mice required for experimental need to test our specific hypotheses, making sure that where possible we use pilot data to determine this in advance. Where possible we will also archive samples for future experimental use (e.g. tissues, faceal samples), to ensure that we gain the maximum possible information from each mouse bred on the licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In applying for the current (Medical Research Council) funding for the proposed work, we used power calculations, incorporating previous data from diabetes-incidence studies in our setting, to determine the minimum number of animals to be used consistent with our

scientific objectives. Going forward, for new studies, we will also use the NC3Rs Experimental Design Assistant to visualise our experiments, and to receive support and feedback on our design.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Prior to beginning any experiments, we will investigate all possible means of reducing the number of animals used, including exploring publicly available data to ensure that our research question cannot be answered without using animals.

We will continue to use a range of methods to optimise the number of animals used:

- we will undertake efficient breeding, ensuring that we avoid breeding diabetes-prone females of 10 weeks or older. This reduces the risk of the female and the litter having to be culled before weaning due to the female becoming diabetic

- we will continue to collect and archive material from mice (e.g. pancreas tissues) that can be used in future studies without having to breed more mice

- we will undertake pilot studies where appropriate to ensure we can make informed decisions about the design of larger experiments

- we will continue to share tissue, as appropriate, with other researchers

- we will refresh our diabetes-prone colony, approximately every 10 generations to avoid genetic drift and to ensure that the diabetes phenotype is maintained at high incidence, since a high incidence of diabetes reduces the number of mice required per study

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be studying mice and our particular focus will be on their development of diabetes.

For this purpose, an important model of spontaneous diabetes is the NOD (non-obese diabetic) mouse, in which, in our colony, 80% of females develop diabetes by 30 weeks of age and <50% of males. No additional intervention is required for diabetes to occur in

these mice, and diabetes monitoring can be achieved by regular non-invasive urine testing. This means that we are usually able to detect diabetes before the mouse displays overt clinical signs.

In addition to studying the standard NOD mouse, we will also study genetic mutations in genes that have been identified as having a potential role in diabetes risk. These mice may have a NOD diabetes- prone genetic background or may have a non-diabetes background, such as a wild-type mouse.

Where more than one mouse line is available with a mutation in a gene of interest, we will select the line most suitable for the purpose, carefully considering the potential harmful impact of each mutation.

In addition to monitoring these genetically-modified mice to determine whether the genetic mutation has had an impact on diabetes development, we may also investigate the function of the immune system in these mice or study the impact of altering the microbes (e.g. bacteria) in the gut on the age at which diabetes develops.

The models that we will use to investigate the immune system will involve using specific substances to target particular immune pathways. This is an alternative to infecting an animal with a live virus or bacteria and is less likely to result in side-effects that make the animal feel unwell. Similarly, when we undertake work to alter the microbes in the gut, we will use the least invasive and harmful method possible - for example administering substances in food or sweetened drinking water.

Why can’t you use animals that are less sentient?

The mouse is the lowest sentient species in which diabetes mellitus can be straightforwardly induced or monitored.

The less sentient species also do not have some of the genes of interest that we =need to study, as they are specific to mammals.

As we are studying diabetes mellitus - a disease which develops over several weeks to months, it is not possible to undertake this work on animals that have been terminally anaesthetised.

We are also interested in the immune system, diabetes risk and the microbes (e.g. bacteria) that live in the gut, and the relationship between these elements. This is only possible to study in a live organism that has an immune system similar to humans, and from which we can collect samples of faeces.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have developed and refined a protocol for care and monitoring of diabetes-prone mice. This includes regular urine testing (which is less invasive than blood sampling) and allows early identification of diabetic mice, so they may be humanely killed before they develop clinical signs.

For the investigation of the immune system, we will use the method that causes the least pain, distress, suffering or lasting harm and that allows us to achieve our objectives. For example, we will administer substances in drinking water where possible, and if a substance needs to be injected, we will use a sub-cutaneous injection where feasible. We will base doses and routes of injection on published resources and pilot work.

When altering the gut microbes, there are several published methods to do this, and we will identify the most effective route to achieve this alteration whilst also minimising harm and distress to the mice. For example we will avoid gavage where a substance (e.g. probiotics) may instead be administered through drinking water. Where a procedure has taken place that could result in harm to the individuals, we will undertake very close monitoring during the immediate post-intervention phase. When introducing a new procedure into our work, we will undertake a pilot study, typically with 1-3 animals, to check that our expectations regarding potential harms are correct.

When administering substances in food or water, we will observe the mice closely to ensure these have not become unpalatable and will intervene (e.g. with sweetener in water) if necessary.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use a range of resources when conducting our experiments - local and national.

Specifically, the published resources we will use include the ARRIVE guidelines, the Laboratory Animal Science Association (LASA) best practice guidelines and the NC3Rs Experimental Design Assistant.

In addition we use, and regularly update, our own Standard Operating Procedures for care and maintenance of mice with specific genetic modifications. We also typically use and discuss experimental planning sheets prior to embarking on experiments, including consultation with the animal welfare officer where relevant, so that all possible refinements are considered before work begins.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Members of the group attend and participate in internal animal welfare meetings at which projects are discussed in terms of advances made in the 3R’s.

We also have representation at local AWERB level which again give us the opportunity to gain information from named people and other investigators. We also attend an internal 3R's day at the establishment.

Information can also be accessed via the establishment's intranet and we will also use relevant web resources e.g. www.nc3rs.org.uk and https://science.rspca.org.uk

We also have access to an NC3R's regional manager.

89. Production of Antibodies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Immunisation, Monoclonal antibodies, Immunoassays, Drug discovery

Animal types	Life stages
Mice	adult
Rats	adult
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim is to immunise animals to generate antibodies as therapeutic agents or as tools to support drug discovery projects.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Antibodies have a variety of roles in drug discovery. They may be therapeutic entities in their own right or they might be tool reagents to validate a drug target or provide immunoassays to support drug development.

What outputs do you think you will see at the end of this project?

Outputs will be antibodies of appropriate functional and biophysical properties to support early drug discovery projects and to facilitate transition from the research to clinical development.

Who or what will benefit from these outputs, and how?

Patients with severe diseases and current unmet need mainly in the area of immunology.

How will you look to maximise the outputs of this work?

Data supporting drug development will be shared with regulators and disseminated at conferences or publication.

Projects that are unsuccessful may be evaluated for publication. Evaluation will look at the risk benefits in terms of whether publication will result in significant added value to the medical literature and whether any additional animal work might be required to complete a publication.

Species and numbers of animals expected to be used

- Mice: 3000
- Rats: 300
- Rabbits: 300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mammals (rabbits, rats, mice) will be used as these have a mature immune system necessary for the generation of antibodies. We also have experience humanising antibodies from these species for development as therapeutic antibodies. Genetically modified animals may also be used. We have acquired a licence to access mice that produce fully human antibodies with the potential to greatly reduce development time for therapeutic antibodies (by removing the need for humanisation).

Additionally, other genetically modified animals may provide a background that enhances the raising of antibodies to a particular target (eg knock out animals whose immune systems have never seen the target immunogen or hyperimmune mice). Having a variety

of immunisation species and mouse strains increases the chances of discovering rare antibodies with very specific functional properties. These might be developed as therapeutics or as a variety of tools to support drug discovery projects.

Typically, what will be done to an animal used in your project?

Animals will be immunised to raise antibodies to the target antigen. Animals may then be boosted to amplify the immune response. Immune enhancing agents may be administered with the immunisations. Periodically, blood samples will be taken to assess the antibody response.

Immunisation rounds and bleeds may be repeated over a maximum of 6 months. At the end of the protocol animals will be killed in order to harvest immune tissues (usually blood, spleen or bone marrow) from which B cells are cultured and antibodies cloned through molecular biology.

What are the expected impacts and/or adverse effects for the animals during your project?

Immunisation and removal of blood will stress animals and give transitory pain. Anaesthesia may be used where the impact of the anaesthesia (stress of induction and transient disorientation afterwards) are considered less than conducting the procedure without. Immune enhancing agents may include Complete Freund’s adjuvant (CFA), that may be used just once, and will cause inflammation at the injected site. We do not expect to see signs of discomfort in animals when CFA is injected subcutaneously according to protocol, but animals are monitored for any signs of ulceration.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The licence has a single protocol with a severity limit of mild, and all animals are expected to be reported as such.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Immunisation results in formation of antibodies that become naturally affinity matured and become more diverse as the immune system is boosted. Immunisation also results in natural antibody molecules that have favourable biophysical properties as a starting point for antibody engineering and/or for long term storage and production at a variety of scales.

Which non-animal alternatives did you consider for use in this project?

Display technologies (phage and yeast). We have access to these technologies in house. Commercially sourced antibodies.

Why were they not suitable?

Immunisations are accompanied by a request form where non-animal alternatives are addressed case by case for each antigen. Reasons not to use commercial antibodies might include non-availability, inappropriate function, poor purity, unavailability at scale or intellectual property. We generally use display and immunisation techniques simultaneously with a specific justification required if a display campaign is not to be initiated. We find display and immunisation complementary in that they increase the diversity of antibodies at our disposal, but immunisation has been consistently more successful in finding rare functional antibodies with favourable biophysical properties. Display technologies are also challenging for cell surface targets. Within the field of therapeutics, non-animal-derived antibodies cannot currently compete, and the vast majority of approved therapeutic antibodies are from animal- derived origin (Custers & Steyaert, 2020).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers have been estimated from the number of projects we expect to support and the typical immunisation campaigns that are required to support them. Rabbits have generally been favoured as their immune system is distinct from mouse and human (the species most often providing the immunogen) and have been a reliable source of antibodies in the past. We expect to conduct many of our immunisations against human immunogens in mice as we have acquired a licence to access mice that can generate fully human antibodies. Rats only tend to be used if more diversity in antibody responses is required beyond what has been achieved with rabbit and mouse immunisations. At the end of the immunisation period, immune tissues are harvested. It follows that the larger the species

the more material is available, and this is a major consideration for numbers of animals that need to be immunised for each antigen in a given species (mice>rats>rabbits).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We expect in most circumstances than an immunised animal will generate an immune response, although the quality of the response may be different between animals. Animal numbers are therefore typically low and determined more by the amounts of immune material that can be harvested from each species than by a statistical evaluation. More animals are used when immunising for a therapeutic antibody (because of the need to achieve antibody diversity to identify rare functional antibodies), such a campaign may start with 2-3 rabbits and groups of 5-10 mice for each immunogen. There may be follow up as needed with groups of 3-5 rats. There is not much room to reduce numbers for a therapeutic campaign, but for non-therapeutic projects we might start with 1-2 rabbits and only consider further immunisations as necessary.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Although we do not breed animals at our facility, we do carefully monitor colonies of genetically modified animals held for us to ensure that there is not a surplus of animals.

We have a crystallography capability which when combined with computer modelling has sometimes allowed existing antibody sequences to be optimised without the need for further immunisation. Whilst this technology is in its' infancy, it is expected it will increasingly add value over the course of this licence. We're also exploring the use of next generation sequencing together with artificial intelligence to predict from an antibody sequence other sequences that might be consistent with functionality. This has the potential to more deeply mine the immune repertoire and to reduce numbers of animals that need to be immunised.

Increasing the efficiency of immunisations, especially against highly homologous immunogens, has the potential to reduce the number of immunisation campaigns and therefore animals used. When consistent with the authorisation of the licence, new immunisation techniques will be evaluated in small pilot studies to assess whether they are more efficient than our existing immunisation strategies. If such developments are outside of the scope of the current licence, amendment may be sought.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

There is not a choice of models for immunisation as they all work in essentially the same way by exposing an animal to repeated doses of an immunogen in order to generate an immune response. We believe that with best practice we can conduct all our experiments within a mild severity limit.

Why can’t you use animals that are less sentient?

The generation of antibodies (or fully human antibodies in genetically modified mice) requires a mature adult immune system. It takes weeks to generate functional antibodies so it is not possible to use terminal anaesthesia for the procedure.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Wherever possible animals will be sourced locally to minimise stress of transportation. All species will be socially housed where possible. Our experience of immunisation is that females tend to give equivalent or better antibody responses than males. We will therefore use female animals in preference to males, and this will minimise injury and stress brought about by fighting. We will only use males (and females) from colonies of genetically modified animals in order to make best use of the colonies and reduce numbers of animals that might otherwise be killed.

Rabbits come into the Special Facility in batches of up to six and will be housed in pens containing a variety of environmental enrichment including pipes, sticks balls and hay, and supplements to the dietary chow. We acclimatise rabbits for a minimum of 2 weeks. Body weights will be recorded weekly, and this will help with getting animals used to handling. Only if an animal is bullied or showing signs sickness will it be removed to single housing.

The rodents will have plastic or cardboard housing, shredded paper, balls (rats) and exercise wheels (mice) and will be acclimatised for at least a week before going on procedure.

The protocol is mild, but animals will be monitored after each procedure to ensure they are fully recovered before being returned to their holding room. We will assess antibody titres over the course of the immunisation protocol. If animals have mounted a strong immune response, they may be taken off the protocol early to spare further rounds of immunisations and bleeds. There is an insistence on a high level of quality control (eg low endotoxin and sterility) of any immunogen injected into animals.

New immunisation techniques may impact positively for animal welfare (eg reduced need for adjuvants). When consistent with the authorisation of the licence, new immunisation techniques might be evaluated in small pilot studies to assess whether they provide refinement over our existing

immunisation strategies. If such developments are outside of the scope of the current licence, amendment may be sought.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Home Office guidance – Antibody Production: Principles For protocols Of Minimum Severity (2000).

ARRIVE guidelines https://arriveguidelines.org/ NC3Rs

https://www.nc3rs.org.uk/ Norecopa https://norecopa.no/ FELASA

https://felasa.eu/ LASA animal welfare https://www.lasa.co.uk/

Recommended dose volumes - Diehl et al 2001

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

There are many 3Rs resources available. There are internal forums (AWERB, Animal Users Committee, Annual 3Rs Poster competition) and outside forums such as NC3Rs, norecopa, FELASA and LASA (both website and conference attendance). Specific to the procedures in this licence, there will be an internal Immunisation Team to consider 3Rs initiatives and emerging immunisation literature. The licence will conform with Home Office guidance – Antibody Production: Principles For protocols Of Minimum Severity (2000).

90. Determining the Tissue Specificity of Cancer Genes

Project duration

3 years 0 months

Project purpose

- Basic research

Key words

cancer, hypoxia, lineage tracing, DNA repair pathways, metabolism

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to determine why genes expressed in all cells of the body only cause cancer in specific tissues and to do this we will study one such gene, VHL (The Von Hippel–Lindau tumour suppressor gene). VHL protein (pVHL) has many important roles especially targeting Hypoxia- inducible factor (HIF), vital for how our body adapts to low oxygen levels, for breakdown. Importantly, when VHL is mutated or deleted it initiates the most common form of kidney cancer.

To achieve our aim we will delete the VHL gene in a novel mouse model in which a fluorescent marker (flag) lights up in cells when the gene is lost (lineage tracer gene). We can then accurately identify and follow those cells, and their daughters (if the cell divides), in which the gene is no longer functioning properly. We will be able to follow the earliest events that result from this gene loss and can try to understand whether, and how, different oxygen concentrations, repair pathways and/or altered metabolism affect the development and progression of cancer so that we can refine cancer therapies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Determining the tissue specificity of cancer is a fundamental question for which we have no good answers. Cancer is caused by changes to genes (mutations) that control how our cells function, especially how they divide, leading to uncontrolled growth of abnormal cells in the body. The mutations that cause cancer can be inherited from our parents, or they can arise from mistakes that occur as cells divide, or because of environmental influences. Many cancers have a unique combination of genetic changes some of which accrue as the cancer grows and even within the same tumour, different cells may have different genetic changes. However, some of these changes may be a consequence of the cancer, rather than its cause. Many of the mutations affect genes that work in all cells in the body, but they only give rise to cancers in specific tissues. If we understood why genes that are expressed in all cells in the body drive cancer in specific tissues we might be able to define routes to therapy for cancers including some for which there is currently no treatment at all (such as in some inherited syndromes).

Also, by using a novel mouse model in which a fluorescent marker (flag) lights up in cells when the gene is lost (lineage tracer gene) and reducing the amounts of agents to delete the gene we can study small numbers of cells with gene mutations or deletions more accurately than previously published work and model better the events in man.

What outputs do you think you will see at the end of this project?

The outputs of this project are:

- To determine accurately what happens over time to cells in different tissues following loss of specific genes that are expressed in all cells. Initially we will focus on the tumour suppressor von Hippel-Lindau (VHL) which is the main driver for kidney cancer and is mutated in an inherited syndrome and will use genotypes that mimic those in the VHL patients to follow lineage marked cells.
- To gain insights into the relative roles and interplay of hypoxia, DNA repair pathways, the immune system and metabolism in the survival of cells following loss of genes that are expressed in every cell in the body, particularly in the development of kidney cancer
- To extrapolate our findings to other cancers
- To rethink and assess the use of ‘field changes’ when deleting genes especially for cancer research (e.g. the use of large and repeated doses of Tamoxifen) and instead to use low doses of Tamoxifen (1/10th to 1/100th of normal dose) to better mimic human sporadic cancer.
- To describe our findings in peer-reviewed publications and scientific meetings
- To describe our findings through public engagement

Who or what will benefit from these outputs, and how?

In the short term, the scientific community will benefit from our findings. We believe that our work will give new insights into the cellular and tissue contexts that allow development of cancer and that this basic knowledge will inform new clinical therapies for cancer, including for individuals with inherited syndromes.

We think that the paradigm of novel lineage marking transgenes to delete and mark small numbers of cells, rather than 'field changes', mimics more accurately the situation in human disease and will change the way in which future animal work is conducted. This would benefit both the animals used in experiments and improve the science.

While we hope to begin to publish papers in the near future all of these impacts will be dependent on the publication and dissemination of the research and are unlikely to occur before the end of the application period (approximately 3 years).

How will you look to maximise the outputs of this work?

The outputs of our work are, and will continue to be, maximised by dialogue and collaborations with scientific and clinical colleagues in a variety of specialities. For example we work on a daily basis with scientists, clinicians, statisticians and bioinformaticians who work with cancer patients, tissue biopsies and human cell lines.

New knowledge will be communicated as early as possible through open access journals to allow access of our findings to everyone.

Species and numbers of animals expected to be used

- Mice: 13,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The animals used in these experiments will be mice, which are the most appropriate fully developed model for studying sporadic and genetically inherited cancers - accurate in important respects, but the least sentient. These animals will be genetically modified strains, carrying induced, targeted mutations or transgenes. We will be using mainly adults in which gene deletion is induced. Occasionally we will use neonates or foetuses because we also want to understand the significance of the timing of gene mutations/deletions on the different cell types and the subsequent development of cancer.

Typically, what will be done to an animal used in your project?

Mice will be bred, and tissue taken for both identification and to determine their genetic status. Mice of the appropriate genetic status will be dosed with a gene deleting agent typically by the oral gavage route and then by a cell marking agent via injection or in drinking water. A cohort of animals will also be exposed to altered gas concentrations for a short period of around one week. Other cohorts will be treated with one of a group of substances to monitor or preferentially kill cells lacking tumour suppressor gene function and therefore interfere with tumour development. After these procedures mice will be humanely killed and multiple tissues harvested for further analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Some experiments (50%) will cause either no adverse effects or only mild adverse effects. Occasionally mice will experience moderate harmful side effects, such as weight loss, or transient changes in behaviour due to the deletion of specific genes (50%). A subset of animals (<10%) will be aged to approximately to one year and may develop tumours. Such animals will be monitored carefully and the animals killed before the tumours can cause distress. Most of the genetic combinations we are using are not expected to give rise to tumours especially since we are interested in observing the early consequences of gene loss that alter cell biology and support or prevent the survival of cells.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

10% subthreshold
40% mild
50% moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are interested in the processes that determine why cancers (both sporadic and those arising from genetically inherited disorders) driven by genes expressed in all cells in the body are tissue specific. Currently it is not possible to study in depth the genetics,

processes and interactions across multiple tissues over a period of time using non-animal approaches.

It is not possible to study all the complexities of either oncogenesis or the altered metabolism associated with loss of these genes within cells of different tissues in anything other than a mammalian system such as the mouse. Furthermore, the mouse model remains the best mammalian system in which to test genetics and understand the downstream complexities of loss of these genes and to allow for translation to medical application where integrative effects on multiple cell types and organ systems can be assessed in the context of any homeostatic mechanisms.

Which non-animal alternatives did you consider for use in this project?

We have used and will continue to use human cell lines and both primary and archived human tumour material, obtained with relevant ethical approvals, to investigate the consequences for different cells of loss of the activity of specific genes known to cause cancer (e.g. VHL, known to be the main driver mutation in clear cell renal cancer). These studies are ongoing in our laboratory. We will also pursue approaches that do not involve animal work using cell cultures, co-cultures and isolated tissues ex vivo to study loss of the genes of interest. We will continue to collaborate with other scientists who have particular relevant expertise e.g. in Systems Biology.

Why were they not suitable?

Human cell lines are already immortalised in some way and therefore do not allow us to investigate the loss of gene function in perfectly normal cells.

We wish to investigate the earliest events (including loss of cells) that may occur when the function of a gene is lost. While studies with human tumour material and adjacent normal tissue are useful these only allow us to see late events in cancer cells compared to normal cells. Also, we only receive small tissue samples and infrequently.

Since we want to determine the reasons why a gene expressed in all cells of the body only gives rise to specific cancers, we need to be able to analyse multiple tissues from a single source. Furthermore,

we want to ascertain whether constraints such as DNA repair pathways, hypoxia and metabolism impact on this process. Currently mouse models are the only system in which to answer these questions.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is based on experience over decades of working with transgenic mice carrying some of the alleles to be used in these experiments. We have also conducted some validation and pilot experiments on another project using a lineage reporter mouse line that we have had made.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the smallest possible group sizes that are still informative for a thorough statistical analysis and logical progression to the next step. Advice is sought, and we use various tools and experts in statistics to support and inform us in these aspects of the programme - for example the NC3R's Experimental Design Assistant and attending seminars such as 'Efficient management of genetically altered mouse colonies'. Also, statistical support is available to the project through members of the group, members of the Department and elsewhere in the University (e.g. Centre for Statistics in Medicine). We are very careful to reduce variation by housing the mice together where possible and ensuring that they are genetically identical. We conduct our experiments in compliance with the ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We optimise our colony management to keep breeding cages to a minimum and use a single database to assist us in tracking and managing the breeding strategy. We use ANOVA and other statistical tools to maximise our ability to detect difference in groups with uneven numbers. Where relevant we use small pilot studies to ensure that projects are feasible and to reinforce our ability to detect difference.

These pilot experiments have allowed us consequently to focus on specific genotypes to study and to refine our breeding accordingly. We share tissues to make the most of mice and harvest multiple tissues from a single mouse for analyses over a number of platforms. This also adds power to our results.

Generating new mouse reporter models, where the reporter is in integral to the original construct, reduces the initial breeding required compared to conventional lineage lines which rely on two lines

initially. Also, importantly such reporter lines improve the accuracy of the data. In a single mouse model with multiple alleles these are maintained as homozygous to reduce numbers of mice.

We have a policy of cryopreservation of all lines and this allows us to remove lines when they are not being used.

Since our mouse lines are genetically identical, where possible we integrate controls across multiple lines to reduce the numbers that need to be bred.

We have had mice made by commercial companies and where possible buy new lines (increasingly as frozen embryos).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The majority of our experiments require us to breed and then compare tissues from mice that are normal (wild-type) or genetically targeted to recapitulate human disease and especially cancer. We hope that these experiments will help us to establish the role of specific proteins and biochemical pathways that determine the tissue specificity of cancers related to certain genes. These pathways are perturbed in both sporadic and genetically inherited human cancers and may potentially be targets for therapy. The breeding protocols cause minimal or no distress. Our experiments are designed to deliver statistically significant results in the least harmful way by making power calculations and focusing on only measurable differences in well matched mice and controls.

We are particularly interested in the earliest cellular consequences following loss or disruption of specific genes and for this purpose have generated a novel mouse model in which there is a reporter integral to the gene construct for more accurate identification of cells where the gene has been deleted. Also, we are aiming to study only small numbers of 'targeted' cells in specific tissues more akin to the picture known to occur in man. Hence we will aim to use lower doses of agents, such as Tamoxifen, than are used routinely to delete genes (in some cases 1/10th to 1/100th of normal doses). These will be delivered by the most humane methods, generally by gavage and where possible by one administration. Most analyses will be conducted on tissues harvested from the mice over a number of different platforms.

We continually seek ways to improve all our animal work and to reduce the level of suffering to the animals.

Why can't you use animals that are less sentient?

Mice are the least sentient animals available with multiple tools, including transgenic and inducible knockout strains, for studying the early stages of cancer that invoke complex cellular, humoral and immunological responses that are similar to humans. Furthermore, although we are aiming to study the earliest events following loss of a gene nevertheless we will need to observe live individual animals over several days or weeks and to be able to determine the role in different tissues of factors such as DNA repair pathways, hypoxia and metabolism that allow cells to develop to cancer. This is not possible in animals that are less sentient.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Potential refinements to reduce welfare costs to the animals include:

- enhanced monitoring, for example, when animals are under hypoxia they will be monitored (including measuring body weights) once daily and animals provided with mash at cage floor level if losing >10% body weight
- providing mash at cage floor level to all animals after oral gavage
- use of conditionally inactivated transgenic animal models to avoid adverse effects of ubiquitous gene deletion in development
- use of a specially constructed transgenic mouse model in which a fluorescent signal indicates if any conditional genes have been deleted has allowed us to reduce the amount of tamoxifen to at least one tenth and a single dose for some recombination genes
- endeavouring to provide extra enrichment and housing to animals if they have to be singly housed

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow published protocols which are assessed by our AWERBs in consultation with our veterinary surgeons. Some of the other resources we use include www.nc3rs.org.uk; https://norecopa.no (including the Experimental Design Assistant and the ARRIVE guidelines) and https://www.lasa.co.uk. We will also use any newly published guidance from the establishment Named Information Officer and Named Animal Care and Welfare Officer or through attendance at local events discussing 3Rs which occur on a regular basis.

We endeavour to use 'blinding' and randomisation in our experiments where possible by enlisting the help of technicians and different members of the research group for various aspects of our experiments. We also use codes for animals in our experiments which do not identify specific genotypes.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We follow and contribute to our local NC3R committee and attend committees for local users, where we share data and methods. We follow activity at www.nc3rs.org.uk and https://science.rspca.org.uk. We will follow best practice as it evolves. We monitor our animals closely. We will exchange information on animal welfare by engaging with animal welfare officers, Veterinary Surgeons and other investigators in our institute and outside it. We seek advice from our colleagues. We keep careful records so we can refine our protocols to be more efficient over time. We keep our training up to date and adopt new methods when they will improve animal care.

91. Investigating Non-Invasive Neuromodulation by Ultrasound for Neurological Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Brain, Transcranial, Neuromodulation, Neurodegeneration, Ultrasound

Animal types	Life stages
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to determine how brief pulses of ultrasound, when directed across the skull, can change brain function, and how this might ultimately be used as a treatment for neurological diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Brain diseases represent a major cause of disability and death in humans. Indeed, current estimates suggest neurological disorders are the second leading cause of death globally. In particular, neurodegenerative diseases are considered a major health challenge as for many disorders age is a major risk factor, and as a society, the proportion of the population of older age is increasing. In Europe alone, 7.8 million people are affected by Alzheimer's disease and 828,000 people are affected by Parkinson's Disease. These diseases are not just profoundly impactful on the individual's quality of life, but also carry significant societal implications. Indeed, in the case of Alzheimer's disease alone, the combined costs of formal and informal care in Europe are thought to be in the region of EUR 160 billion annually. Crucially, there are currently no curing treatments for these conditions, despite many years of pharmaceutical research.

Over many decades of research, sophisticated tools to change the function of the human brain have been developed, and are now used in experimental and therapeutic contexts. From pharmacological approaches to direct electrical stimulation of the brain, various tools are available to us to control brain activity. These have allowed us to better understand the contributions different areas of the brain make to cognitive tasks and provided us with a means to treat neurological disorders. However, these approaches have significant limitations. Drugs have side effects and often modest clinical effects when used therapeutically. Furthermore, whilst direct electrical stimulation of the brain does provide unparalleled target accuracy over where and what in the brain is being regulated, techniques such as deep brain stimulation are invasive and carrying significant surgical risk. Non-invasive ways to affect brain function, that do not require drugs or surgery, such as transcranial magnetic/direct current stimulation, serve as a potential alternative to regulate brain activity. However, they suffer from relatively poor spatial precision. Considering these shortcomings, the development of a non-invasive and spatially precise method to change brain function would be extremely beneficial and could open new avenues for therapies for neurological diseases.

Ultrasound is traditionally associated with medical diagnostic imaging, where it is used to visualise anatomical structures. At the pulse frequencies employed, this type of ultrasound does not induce detectable biological responses or adverse effects and is therefore considered a non-invasive diagnostic procedure. When ultrasound pulses are delivered across the skull at low intensities, however, brain activity can be modified and cognitive effects induced. My group has pioneered developments in the understanding of the genetic, molecular and cellular consequences of ultrasound neuromodulation on the function of brain cells. What is now urgently required is a systematic investigation into the potential therapeutic applications of the novel technique, to establish when, where and how this intervention could be used for medical benefit. The purpose of the studies outlined here is therefore to accelerate and facilitate the development of transcranial ultrasound stimulation as a treatment for brain diseases by characterising the fundamental physiological consequences of ultrasound-mediated neuromodulation.

What outputs do you think you will see at the end of this project?

The main outputs of this study will be the data and scientific publications relevant to the efficacy of transcranial ultrasound stimulation which is essential to determine the potential of this treatment for translation to a human clinical setting.

Who or what will benefit from these outputs, and how?

In the short-term, we will establish critical information concerning the application of ultrasound as a brain-modifying technique. This will occur throughout the life of the project. This will benefit other researchers and engineers in the field who will use our findings to inform their work and optimise the development of ultrasound devices. If the study proves successful, in the medium-term it is expected to facilitate the translation of transcranial ultrasound neuromodulation into human clinical trials and in the long-term benefit patients with a range of neurological conditions for which neuromodulation is a useful therapeutic intervention.

How will you look to maximise the outputs of this work?

The findings of the work will be submitted for publication in peer reviewed scientific journals and presented at national and international conferences. In addition, I will use my existing links with medical companies developing ultrasound devices to disseminate the findings to commercial stakeholders.

Species and numbers of animals expected to be used

- Rats: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rats will be used for the outline work as the experiments require animals with a brain anatomically similar to that of humans and of sufficient size to enable specific regions to be targeted using transcranial ultrasound. Mice are unsuitable as their brain is too small to enable effective regional targeting. In addition, the limited cognitive ability and behavioural repertiore of mice make them unsuitable for some of the assessment tasks needed to determine the effects of transcranial ultrasound stimulation on memory and cognition.

Adult rats will be used in order to reflect the developmental stage of human patients on whom transcranial ultrasound is most likely to be used.

Typically, what will be done to an animal used in your project?

Upon arrival in the unit, the animals will be group-housed and allowed to acclimatise for a week, during this period the animals will be regularly handled to habituate them to the

research staff. For all initial and terminal studies, the animals will be anaesthetised, the fur over the scalp will be removed and the animals place in a stereotactic frame. Ultrasound gel will be applied to the scalp and the ultrasound probe accurately aligned over the target brain region. Ultrasound stimulation will then be applied for between 30 seconds and 5 minutes. Thereafter, the animal will either be immediately killed, in order to harvest brain tissue for analysis or, allowed to recover from the anaesthetic. Recovered animals may undergo repeat stimulation on up to three occasions.

Attempts will also be made to refine the technique by training the animals to allow transcranial ultrasound stimulation to be undertaken whilst fully conscious. Using appetitive positive-reinforcement, the animals will be trained to voluntarily enter and immobilise within a conical tube. Training will involve encouraging the rat to enter a cone shaped tube to obtain a food reward e.g. sucrose water. Once the rat has learned to enter the tube to obtain a reward, the time spent in the tube to obtain the reward will be gradually increased until it is sufficient to allow ultrasound stimulation to be undertaken.

Plan of work

Initial studies will focus on determining the immediate consequences of ultrasound stimulation on brain function. To evaluate this, rats will be killed by decapitation immediately following stimulation and brain tissue harvested for ex-vivo analysis. Thereafter, studies will be undertaken to evaluate the persistence of effects induced by stimulation. In these studies, the time between stimulation and killing will be delayed by a pre-determined amount. Next, studies will be undertaken to determine whether repeated stimulation has a cumulative effect, beyond that of a single stimulation. For these studies, animals will undergo repeated ultrasound stimulation on alternate days, up to a maximum of 3 times (i.e., 3 stimulation sessions, separated by at least 48hrs between each single session).

A subset of animals, in the delayed recovery groups, will undergo non-aversive behavioural testing to assess the effect of stimulation on memory, cognition or behaviour. This is because we are targeting regions of the brain thought to underly these types of behaviours. It will therefore be important to understand what the effects are of stimulation on cognition. The typical tests used for these studies include: T maze, open field or novel object recognition. Testing may occur both before and after ultrasound stimulation, in up to 3 non-regulated cognitive tests, not more than once daily and for no more than 3 months.

What are the expected impacts and/or adverse effects for the animals during your project?

Prior to undertaking any procedures, the animals will be habituated to human contact for a period of at least one week to minimise any stress caused by handling during the study. During anaesthetic induction and recovery, animals may experience mild transient stress, this may be experience by some animals on up to three occasions. Ultrasound stimulation itself, is not expected to have any adverse effects and all animals are expected to recover

uneventfully from the anaesthetic following stimulation and to resume normal behaviour shortly thereafter. The method of killing used causes instantaneous death with no suffering.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Rats

- Mild (70%)
- Non-recovery (30%)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The objectives of this project are to determine the cellular and molecular effects of transcranial ultrasound stimulation on the brain. My group has already performed extensive in vitro experimental work which has established the proof-of-concept for the proposed study. However, our in vitro work does have fundamental practical limitations that prevent further progression towards our main objectives of utilising ultrasound stimulation to treat degenerative brain diseases. To further progress this work requires the effects of ultrasound stimulation on brain function to be determined. This can only be achieved in studies performed on living animals as a fully functional brain only exists in a living animal.

Which non-animal alternatives did you consider for use in this project?

We also considered characterising neuromodulatory effects in human participants.

Why were they not suitable?

Whilst human studies have been conducted into the effects of transcranial ultrasound stimulation, the field is still very much in its infancy, and critical exploratory work needs to be completed first to determine its efficacy and safety before substantive work can be ethically considered in humans.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated animal numbers have been derived by extrapolating from similar work conducted by others in the field, with consideration given to our extensive in vitro data to predict effect sizes. Using the NC3Rs Experimental Design Assistant has ensured due consideration has been given to the design of the experiments and therefore the necessary animal numbers.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A comprehensive analysis of similar in vivo studies was undertaken to predict the likely effect sizes of the phenomena we aim to study. This was then integrated with our existing data to derive the final expected effect sizes. This has facilitated calculated best-estimate power analyses to determine the minimum number of animals required across the experiments planned to be conducted. The NC3R's Experimental Design Tool has been used in the planning of the proposed experiments. Where scientifically appropriate, data from control animals/experiments obtained in the early phase of the study will be used as the control throughout the entirety of the study.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will undertake multiple parallel assays from the brain tissue we harvest from each animal. Thus, one animal will provide tissue and cell material that can contribute to multiple data sets. This eliminates the need of using one animal per assay. We plan to undertake small-scale pilot studies prior to full- scale investigations in each experimental paradigm. This will allow us to establish ground-truth effect sizes and allow us to adjust our sample sizes with the appropriate statistical analyses.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

During this project, we will use wild-type rats. Transcranial ultrasound stimulation is not expected to have any adverse effects. At the end of the study, the animals will be killed humanely.

Why can't you use animals that are less sentient?

To undertake the outlined studies it is essential to use an animal with a brain that is known to function in a manner similar to that of humans in order to have confidence in the translational potential of the safety and efficacy data generated. Rats have been selected for these studies because the anatomy of their brain is similar to that of humans and is of sufficient size to enable specific regions to be targeted using transcrainial ultrasound. Mice are unsuitable as their brain is too small to enable effective regional targeting. In addition, the limited cognitive ability and behavioural repertiore of mice make them unsuitable for some of the assessment tasks needed to determine the effects of transcranial ultrasound stimulation on memory and cognition.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be group housed and we will implement cage environment enrichment. A major goal of this project will be to establish an appetitive positive reinforcement training regime that allows for voluntary immobilisation of the animal for ultrasound stimulation. Thus, a major refinement, if successful, will be removing the need to administer anaesthetics.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Experiments will be conducted in line with Good Laboratory Practice (GLP) standards. The project will be managed and implemented in accordance to the standards and frameworks defined by the Laboratory Animal Science Association (LASA).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will undertake regular relevant Continuing Professional Development (CPD) to stay informed about advances in the 3Rs. The University Named Information Officer disseminates CPD opportunities on a weekly basis. I will use this to identify courses, webinars and other sources of information on 3Rs developments that I can attend and participate in. In addition, I subscribe to Norecopa's newsletters which contain useful information about developments and implementation of 3Rs principles. My active

engagement in these will be recorded in the University’s electronic training and competency system.

92. Mechanisms and Treatment of Mitochondrial Dna-Related Disorders

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

mitochondrial DNA, energy metabolism, organ pathology, neurodegeneration, therapy

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Understanding and rectifying the pathological consequences of the mitochondrial DNA (mtDNA) dysfunction.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The DNA in mitochondria (mtDNA) is essential for life: hundreds of mutations in mtDNA and scores in nuclear encoded factors required for mtDNA maintenance cause inherited human diseases, which have a collective incidence of 1 in 5000. Currently, there are no effective therapies that limit the high morbidity and mortality of these disorders. Added to the burden of the genetic diseases, are the 'acquired' conditions in which mitochondrial dysfunction is implicated. Thus, a means of preventing, reversing, or mitigating the loss of mitochondrial function represents a major unmet clinical need. In this regard, the project aims to 1) advance our understanding of the factors that control mtDNA maintenance and propagation of mutated mtDNAs and 2) unravel the sequence of events that lead from mitochondrial dysfunction to organ pathology. The new knowledge will lead to 3) design and test new therapeutic approaches to mitigate the pathological consequences of mtDNA dysfunction not only in genetic forms, but also in more common pathologies where mitochondrial dysfunction is implicated.

What outputs do you think you will see at the end of this project?

By the end on the project, we expect to:

1) Discover novel factors regulating the amount (depletion) and quality (deletions and mutations) of the mtDNA

2) Advance our understanding on the cascade of events leading from mtDNA dysfunction to organ pathology in rare and common disease

3) Establish the therapeutic potential of small molecules identified in vitro and successful tested in preliminary experiment in vivo and identify novel and more targeted compounds for mtDNA- related pathologies.

The novel and exciting information will lead to a) publications in leading scientific journals and b) development of credible therapy for mtDNA-related diseases.

Who or what will benefit from these outputs, and how?

The project is research-based with a profound translation potential. As such, it is expected to benefit academic research working on mitochondrial biology and metabolic disorders. Moreover, because mitochondrial DNA dysfunction features the normal ageing process and neurodegenerative disorders, the findings can benefit researchers working in these two broad fields in the UK and internationally.

Finally, the new knowledge will be invaluable for our clinical colleagues who have been to offer anything than symptomatic treatments to date. In this respect, once established the compounds are effective at restoring biochemical, molecular and phenotypic manifestation in mice, we plan to

a) securing IP for novel compounds

b) perform experimental medicine studies (from 2-5 years)

How will you look to maximise the outputs of this work?

All the knowledge generated through this project will be made available to the scientific community through its publication in peer-reviewed leading journals, as well as its dissemination at scientific meetings and conferences. Also, unsuccessful, or inconclusive approaches will be made available through its publication in journals which facilitate the disclosure of negative results. Moreover, all the biological samples generated during this project will be offered to collaborators for their use in other research topics, thus maximizing the outputs from our work and reducing the number of animals used in other projects.

Species and numbers of animals expected to be used

- Mice: 6400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The current project is intended to use GA mice modelling mtDNA human disease and their correspondent WT animals. Some of the biochemical and phenotypic manifestations resulting from mtDNA dysfunction occur after many years in patients and several months on GA mice, thus requiring the animal study to be extended from very early (neonate-juvenile) to late life (aged animals) stages.

Typically, what will be done to an animal used in your project?

Mice will be bred and maintained under standard conditions following approved procedures at the housing facility. Some of the animals will receive daily treatment with drug candidates aiming to restore mtDNA metabolism or purge pathological mtDNA variants (with a variable duration ranging from 1 to 30 weeks). Peripheral blood samples and biopsies of superficial tissues may be collected, and behavioural tests performed to understand the course-time of the pathology and the effectiveness of any given treatment without the need for killing the animal. At the end point of each experiment, mice will be humanely killed by approved methods and relevant tissues and/or organs collected for downstream analysis. Some mice may have their diet modified (eg: high fat or aminoacid deficient diet) to better mimicking human pathology and to gain insight into the role of mtDNA in rewiring the metabolism, which appears to be common to mitochondrial dysfunction. During all the experiments, any animal showing any unexpected harmful phenotype will be humanely killing to avoid unnecessary suffering.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of the GA mice used in this project will not experience adverse effect greater than mild severity. Some mice may progress to moderate severity in late stages of life (after 12 months of age), displaying body weight loss and motor impairment. If this is the case, animals will be transferred to a specific protocol with moderate severity and carefully monitored.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

It is expected that the vast majority of the GA mice used in this project (85%) will experience mild severity. Only a subset (15%) of the animals might reach moderate severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The research questions that this project aims to address require the study of multiple cell types and organs, and in some cases how the dysfunction of one tissue can affect the others. Thus, despite the advance in the developing of in vitro and in silico models, they fail in modelling the huge complexity of the in vivo pathology.

Which non-animal alternatives did you consider for use in this project?

The in vivo experiments proposed in this project follow extensive work performed in vitro (cell culture) models, which provide critical direction for the animal work. Whenever possible, we plan to continue in vitro approaches in order to avoid and/or to refine our animal experiments.

Why were they not suitable?

While cell culture models can provide invaluable insight into molecular and biochemical disease mechanism, they fail in modelling the huge complexity of the in vivo pathology.

Thus, to proper mimicking and understanding human disorders, the study of animal models is required..

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimation of the number of animals has been done based on the requirements for the maintenance of the different mouse lines and the number of animals required for the experiments detailed in this project licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our studies will be designed following PREPARE guidelines (https://norecopa.no/prepare) and considering the available online tools at NC3R's website (https://eda.nc3rs.org.uk/). We have worked closely with statisticians to plan studies to ensure the minimum number of animals are used, and will continue to use power calculations, randomization and blinding where appropriate to obtain statistically relevant results from the smallest group size possible.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Careful breeding will be undertaken to minimise the numbers of animals bred compatible with the scientific purpose. Where large cohorts of animals are required for experiments, the mice will be bred intermittently to generate age-matched cohorts. Also, when possible, the mice bred and maintained to secure the colony will also be used to provide experimental material. Experiments will be co-ordinated to allow overlapping projects to make use of the tissue samples obtained from these studies, thus reducing the number of animals used. Also, as much post-mortem tissues as possible will be collected and properly stored from each mouse (despite not being used in the short-term), to be offered to collaborators and/or to be used in future studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mouse is the most versatile and widely used vertebrate species for modelling human genetic diseases and thus for translational research, which forms the bulk of the work proposed in this project licence. In mtDNA-related disorders, multiple organs are involved, and the complex interconnections between them lead to the final disease phenotype. Thus, the study of an animal model of these disorders is crucial.

Why can’t you use animals that are less sentient?

Mouse models of mitochondrial DNA diseases are the closest to human pathology. Despite several models of mtDNA disfunction have been reported in less sentient vertebrate species (i.e. zebrafish), they fail in mimicking human disease, being murine models the only ones where the affected organs are relevant for human mitochondrial pathology. Also, the use of mice allows to access to a wide range of genetic assays and test kits that are not available for other species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

When breeding, genotyping will be performed on ear biopsy samples, allowing us to reuse the tissue removed for the purposes of identification and avoiding extra suffering than that resulting from normal husbandry practice. Unavoidably, some mice will be singly housed e.g. when presenting aggressive behaviour or animals out-living their cage-mates. When possible, single housed females will be combined with compatible cage-mates. Regarding compound administration, the preferable route of administration will be always oral and in the less stressful way, e.g. replacing oral gavage for drinking water whenever possible.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow LASA Good Practice Guidelines (https://www.lasa.co.uk) and/or resources available at NC3R's website (https://www.nc3rs.org.uk/3rs-resources).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep informed about advances in the 3Rs through the training courses and activities disseminated by UCL BS Administration as well as by the NC3R's website (https://www.nc3rs.org.uk).

93. Understanding How Metabolism Contributes to Cancer Development, Progression and Treatment Response

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cancer, Metabolism, Metastasis, Microenvironment

Animal types	Life stages
Mice	juvenile, adult, pregnant, embryo, neonate, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand how metabolic changes in both tumour and normal cells can affect different stages of malignant progression, and how to target these changes for cancer therapy.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer (in this protocol we are looking at pancreatic, lung, and intestinal, and possibly others in the future depending on how the project proceeds) is a major cause of premature

death worldwide and treatments for late-stage metastatic disease are urgently needed. This project brings together established and novel systems of metabolic control with the recent appreciation of how host factors control cancer, and how these might be used for therapeutic effect.

What outputs do you think you will see at the end of this project?

The project will produce two broad types of output. Firstly, there will be peer reviewed scientific publications that will contribute to the general pool of knowledge about the role of metabolism in cancer (such as pancreatic, lung, liver and intestinal) . This will help to drive the understanding of this area and serve as a platform for further studies in our group and others. These publications will be supported by other means of dissemination of results, such as presentations at international research conferences. A second output will be the identification and validation of new targets for cancer therapy that focus on metabolic pathways. These may lead to the development of new drugs to affect metabolic enzymes or entirely new treatment approaches such as dietary modulation. Several pharmaceutical and biotech companies are already pursuing these avenues and will be well placed to take forward the information generated by the outputs from this project.

Who or what will benefit from these outputs, and how?

In the short term, the key beneficiaries of these outputs will be the general cancer research community, who will be able to build on the data generated through this project to increase our understanding of the mechanisms linking metabolism and cancer. In the mid and long term these outputs will be of benefit to cancer patients and clinicians, through an increased understanding of how to use existing treatments and the development of new therapies. This work is novel and does not seek to reproduce work of others but will be used to build on our previous and ongoing findings.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this work through peer reviewed publication in open access journals, seminars and presentations at international meetings and conferences. Where appropriate, we will work with the communications teams at our Institute to disseminate our results more broadly to the public, for example through press releases and lay talks. We will also maintain close links to industry partners and other external collaborators to ensure the most rapid translation of our work to patient benefit.

Species and numbers of animals expected to be used

- Mice: Mice: We expect to use up to 7,000 mice per year over 5 years. It should be noted that 50% of these will not undergo scientific procedures, but will be used solely for breeding and maintenance of colonies or if appropriate provision of tissues.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project focuses mainly on the use of adult mice to study cancer development, with the occasional use of juvenile mice, for example in the study of dietary interventions.

Mice are the only suitable animals for these studies for a number of reasons.

1. The physiology of cancer in mice is consistent with the human disease. Our aim is to study the interaction of whole-body systems with cancers, and for this it is necessary to use a mammalian system that mimics these aspects of human physiology.

2. Mice are routinely used to model cancer in a whole animal. As a result, there is a vast body of information and large numbers of models that are used by the whole field. This means the design of our experiments will be informed by previous data, reducing the requirement for preliminary studies.

3. As mice are so widely used, the results from our project will feed into the growing understanding of metabolism in cancer. Our work will be relevant and complementary to other studies, so helping to take forward the entire field.

Typically, what will be done to an animal used in your project?

To study tumour (such as pancreatic, intestinal and lung cancer) development , mice will be exposed to genetic modification (which may be spontaneous or targeted to a specific organ or selected time point in life, such as after birth), known or potential carcinogens and transplantation of tumour cells. These procedures may also involve viral infection.

To study how host responses promote or retard cancer progression, we will use mice of different genetic backgrounds (e.g. to study metabolism we will use obese or diabetic mice) or mice exhibiting co-morbidities , such as infection to induce inflammation, or we will use different diet to induce metabolic changes in the mice to see the effect on tumour development. The effect of aging will also be studied by maintaining the mice for two years.

The effect of different potential treatment options will be assessed using drugs, biological therapies such as antibodies or radiation.

Some mice will receive transplantation of certain immune cells to allow us to modulate and assess the role of the immune response in cancer.

Some mice will be exposed to different diets.

Tumour development will be followed by direct measurement of superficial tumours or non-invasive imaging of internal tumours, in some cases using genetic marking with a reporter

protein such as iRFP (a fluorescence protein that will enable us to observe the tissues in-situ).

Tissues and fluids will be collected from these mice, either from live mice (e.g.blood, urine or faeces collection) or post-mortem (e.g. tumour and other organs).

The duration of these experiments will vary considerably depending on the model, ranging from a few days to years. In most cases we have experience and knowledge of the expected timeframes and experiments will only be maintained for the minimum duration to address the scientific need.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will experience a range of impacts from extremely mild (for example some dietary changes) to moderate (in the case of aggressive tumour development or infection combined with interventions such as surgery). In the latter case we would expect weight loss and abnormal behaviour such as hunching. While we have significant experience and can predict the time frame and severity of each model, unanticipated events can occur and all mice will be monitored closely for signs of ill health or distress.

Experiments will be terminated if they reach moderate severity limit.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most of the animals in these studies will be expected to develop tumours. The impact and severity of each experiment will differ depending on which tumour model is used, and the aggressiveness of the tumour combined with the efficacy of any potential intervention. We anticipate that the tumour experiments will result in 50% mice reaching moderate severity as a result of repeated procedures. The other 50% will be a combination of mild and sub-threshold as a result of breeding maintenance of the different cohorts.

What will happen to animals at the end of this project?

- Killed
- Used in other projects
-

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our project aims to understand cancer development in the context of the whole body, integrating the interplay between cancer cells and normal hosts systems to determine mechanism of cancer growth and spread, as well as the effects of systemic therapeutic interventions. The complexity of these interactions cannot be measured in tissue culture systems and the mouse provides an ideal mammalian model to carry out this work.

Which non-animal alternatives did you consider for use in this project?

Our studies include the use of tissue culture protocols such as the standard 2D cultures that uses established cell lines, also 3D organoids (from tumour and normal organs, such as liver, pancreas, intestine and lung) and co-culture systems (i.e. culturing two or many different types of cells together to mimic as close as possible to in vivo situations). These will be used extensively to establish hypotheses, understand mechanisms and examine direct interventions in cleaner, simpler systems. For example, we will be culturing adipose tissues and cancer cells together, and see the effect of adipose tissue on cancer growth or other parameters such as migration of cancer cells. The results from these studies will guide the mouse work and reduce the number of in vivo experiments required, and finetune the subsequent in-vivo hypotheses.. We will also regularly search in the literature, and attend meetings for unpublished new data, to see if there are any new methods that offer better non- animal alternatives, should there be any that is applicable to our aims.

Why were they not suitable?

The value of organoid and co-culture systems is constantly improving, and we will continue to modify and upgrade this work over time. However, even the most sophisticated cell culture models cannot reproduce the complexity of the tumour microenvironment, tumour dissemination to distant organs or the effect of systemic whole-body changes on cancer progression. These questions can only be accurately modeled within the context of the whole live animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used in each of the studies is based on extensive (over 10 years) previous experience using the same models, published literature, advice from colleagues using the same models and the advice of our in-house statistical experts . In most cases power calculations will be used to estimate the number if the parameters are known.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

1. All experiments will be designed to use the minimum number of mice required to achieve statistically meaningful results. This will be developed with the advice of in-house statisticians, where required.

2. In all cases, experimental designs will ensure the extraction of maximum data, for example by measuring primary tumour and metastasis in the same animal or harvesting multiple organs from the same mouse for analysis.

3. Tumours in genetically engineered mice tend to develop over broad time scales, making it difficult to know exactly when to harvest mice for the most informative results. Published data on the models and online tools such as NC3Rs Experimental Design Assistant will provide guidance for the optimal number of animals needed to address the questions. Non-invasive imaging techniques or endoscopy will be used where possible to accurately follow tumour growth over time, limiting the number of mice required to hit the appropriate point of tumour development for the study.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

1. We constantly optimize our breeding strategies to minimize the number of animals needed to achieve the desired genotypes for our genetically engineered models. Where possible, orthotopic tumour transplant models will be used, which do not require the breeding of genetically altered animals and use fewer animals per study.

2. Studies from cell culture models will be used to ensure that only the strongest hypotheses are tested in the mouse

3. Animals will be shared between experimental groups, where possible. For example, normal control animals can be obtained from our breeding colonies where they would not normally be used for a study.

4. Where possible we will share tissue from experiments to enable multiple ex-vivo studies

5. Most experiments will be based on previous studies, allowing us to predict the required numbers of animals. .

6. Using advice from HO efficient breeding of GA animals (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773

553/GAA_Framework_Oct_18.pdf) we constantly monitor animal use and archive lines by cryopreservation when not required over a period of time.

7. Where possible, unwanted genotypes from genetical breeding experiments will be utilized for other experimental studies – this is coordinated across the research group prior to starting the mouse study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The project uses genetically engineered mice, models of cancer induction by carcinogens and tumour cell transplantation. Infection models and other models of metabolic syndromes such as obesity and diabetes are also used. In all cases protocols to limit pain and suffering (e.g. ultrasound guided injection to limit surgery) are used. The conditional and inducible nature of many models ensures lesions are targeted to the cell type of interest (if the tissue specific cre allele is available for the tissue of interest) in adults, reducing non-specific off target effects.

None of the protocols exceed moderate severity levels. All mice on procedures are constantly monitored and humanely culled when exhibiting signs of altered health status and /or tumour burden or other specified end point is reached.

Why can’t you use animals that are less sentient?

Less sentient animals do not possess the physiology (e.g organs, hormones, metabolism and immune system) that will allow us to understand the interaction of tumours with these complex host systems which is the focus of our studies. Only mammals can provide the level of complexity required to make these studies relevant to humans. For example, to study effect of insulin signaling that is a key factor in metabolic disease, it is known that cold blooded animals is not a good model as the pathway is not well conserved and their energy metabolism is much different compared to warm blooded animals such as mice and human. Many genes are also not conserved in lower animals as well.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We take advice from the experts within various fields of medical research and share our own experiments of refinements and improvements. We are highly motivated to minimize harms and stress to our mice as this also allows for more accurate and reproducible

experimentation. For example, we have moved to imaging guided transplantation of cancer cells – a technique that avoids surgery. We also follow local NVS policy on post-operative care and pain management. We also increase monitoring of the tumour models to determine the earliest end-point possible, and will use orthotopic models if applicable.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For all studies we will refer to the Guidelines for the welfare and use of animals in cancer research (Workman et al, 2010) and ensure best working practice. We consult the NC3Rs guidelines and monitor refinement where such practices are published (NC3Rs/LASA websites). We also consult the Guiding principles aseptic surgery:https://www.lasa.co.uk/PDF/LASA, and Guiding_Principles_Aseptic_Surgery_2010.2.pdf Refining procedures for the Administration of substances: https://doi.org/10.1258/0023677011911345

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Training programmes in monitoring tumour development are mandatory for all users for each of our models. The trainers are specialists and users in specific tumour models and are shadowed by the trainee during the routine checking of the animals for symptoms. The trainees will be considered trained once the trainers are satisfied with their ability to recognize the signs of tumour development, humane end-points, and what samples to be collected and processed. We received frequent updates and recommendations from our NIO and we have an in-house NC3Rs regional programme manager who will provide guidance to the lab to ensure the latest 3Rs recommendations will be implemented whenever possible. Also, we keep ourselves up to date (e.g. from reading literature and attending conferences) to recent developments regarding better mouse models of various human diseases that are relevant to our study in cancer and metabolism

94. Molecular Analyses of Caspases in Infection and Immunity

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

innate immunity, infection, inflammation, gut, pathogenesis

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim is to better understand the role of the immune system in health and disease. We will investigate how the immune system protects us against infection, and how an over-active immune response contributes to detrimental disease outcomes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The immune system protects us against infectious microorganisms, promotes tissue repair and helps maintains homeostasis. Molecules called the 'inflammasomes' can sense infection, tissue damage or stress. Cells then launch an immediate innate response by releasing many inflammatory mediators called cytokines. Normally, these inflammatory

processes are self-limiting and essential for clearing infections. However, deregulated immune responses are linked to the development of several diseases. We will focus on how the immune response is beneficial during infection, and how its deregulation negatively impacts disease outcomes.

Inflammasomes play important roles in defending against infectious diseases of the gut (e.g., diarrhoea, gastroenteritis) and lung (e.g. pneumonia), as well as non-communicable diseases (e.g., inflammatory bowel disease, neurodegenerative disease, arthritis conditions and heart disease).

Furthermore, mutations in genes encoding inflammasomes are linked to hereditary fever syndromes. Inflammasomes thus affect such a wide-ranging set of organs, tissues, and contribute to different diseases. Previous work in this field has showed that genetically removing inflammasome-associated genes, or pharmacologically blocking them through drugs, reduces inflammation and improves disease outcomes. Indeed, drugs against these pathways (e.g., anti-IL1 therapies) have been approved for use in humans. Current research focuses on finding newer and better therapeutic avenues. This work will complement other in vitro work in the group, and will use wild type or newly derived genetically altered (GA) mice. We hope to shed light on disease pathogenesis and identify potential new therapeutic interventions.

What outputs do you think you will see at the end of this project?

Outputs will include new, fundamental and basic scientific information on the role of innate immune molecules called inflammasomes in infection, sterile inflammation and homeostasis. We will also generate new GA mice that will be of benefit to the wider community of scientists. We could also find out whether newly designed drugs or other substances could serve as starting point for the design of new therapeutics. This information will be disseminated by publications in the scientific literature.

Who or what will benefit from these outputs, and how?

In the short term, our newly derived GA mice will benefit the research community in the field and will be available to other groups nationally and internationally. Our experimental findings will be useful to other basic scientists working in immunology, microbiology, biochemistry, cell biology, among others. In the long-term, our outputs could form the bases of new concepts in mammalian and human immune responses, and future cross-disciplinary work. Therefore, our findings could also inform scientists who undertake translational work more directly connected to the clinic.

How will you look to maximise the outputs of this work?

Peer reviewed research publications and reviews, presentations at conferences, invited seminars and announcements on social media will disseminate our work to groups worldwide.

We will use work-in-progress seminars presented by students and postdocs in the group, my group's participation in the campus-wide collaborative consortia, and interactive Away Days to make our work more widely known.
Species and numbers of animals expected to be used

- Mice: 7300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use inbred strains of wildtype and GA mice for this work. Mice have a proven track-record in the field of immunology and infection because of the presence of both the innate and adaptive immune systems, the latter being absent in invertebrate experimental models. Moreover, like in humans, molecular pathways involving caspases and inflammasomes, which are the main areas of focus in our research, are known to play key roles in immune responses in mice. Our work will therefore build on a robust body of work and provide new insights in this field.

Most work will use adult animals, as they respond robustly to infection and inflammation and provide relevant biological information. We plan to use pregnant animals and embryos only during the derivation of GA mice, or when tissues from non-adult life stages are required for in vitro studies.

Typically, what will be done to an animal used in your project?

Typically, animals will be administered an infectious agent via injection (intraperitoneal (i.p.), intravenous (i.v.), or intratracheal (i.t.)) or orally. Other substances (e.g., transgene-inducing agents, antimicrobials, immunomodulatory agents etc.) may be administered orally or via injection.

In the majority of cases animals will undergo between 3-5 procedures (e.g., typically, 3 oral gavages of transgene-inducing agent over 5-6 days, followed by single administration of infectious agent OR an inflammatory substance, followed by timed killing by a humane method, normally up to 5 days).

Where immunomodulatory substances are tested for their impact on the outcome of microbial infection or inflammation, an additional step may include administration of substances on one or more occasions (e.g. antibiotics in drinking water or i.p. injection of cytokines at timed intervals).

A small number of animals will undergo additional steps, such as those that undergo radiation & reconstitution or those that will be imaged (e.g., experiments using light-emitting microbes, substances or strains of mice) during experiments.

What are the expected impacts and/or adverse effects for the animals during your project?

The infectious agents we will administer cause gut or lung inflammation, and in some cases, invasive disease of the liver, spleen or other organs, leading to overall symptoms such as weight loss, reduced feeding, diarrhoea-like symptoms of soft stools, altered breathing, changes to movement and posture.

The inflammatory substances could trigger localised inflammation in the abdominal cavity and systemic inflammatory symptoms.

Experiments are only expected to reach moderate symptoms and animals will be killed by a humane method at timed intervals that last between hours (e.g. every 6 or 12 h) or days (e.g., 24 h time-points).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity of our experiments is mild to moderate. About 30% animals will experience mild symptoms and 70% moderate symptoms.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Response to infection in the intestine or lung, which contain different cell types and commensal microorganisms cannot be studied in vitro using cell culture systems. Similarly, inflammation that affects multiple tissues and organs, including circulating blood, cannot be recapitulated in vitro. Use of animals for these studies is therefore scientifically justified.

Which non-animal alternatives did you consider for use in this project?

Cell-culture methods, including cell lines and organoids.

Why were they not suitable?

Available cell-line models do not faithfully reflect the behaviour of cells within the natural environment of tissues. Organoid-based are typically only one cell-type and the interaction of epithelial cells with immune cells cannot be studied in these systems. Similarly, the intercommunication between the host and commensal microorganisms cannot be studied in vitro.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals reflects the number necessary to achieve the scientific objectives outlined in the work.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Wherever possible we will use light-emitting reporters that allow non-invasive optical imaging of the same animal throughout the experiment. This avoids the need for additional animals that would have otherwise been required at various stages of the study.

We will seek advice from statisticians and online resources such as the NC3R website, ARRIVE and PREPARE guidelines, and published literature in designing experiments. Wherever possible, we will use randomised block designs that offer higher power for fewer animal numbers. Relying on principles of the 3Rs, the minimum number of total animals required to obtain statistically significant inferences will be used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Wherever possible we will use modelling using software, cell lines, organoids or recombinant proteins for our studies, and avoid obtaining cells/proteins from mice i.e., we will avoid using animals only to provide material for routine work.

Colony management will ensure breeding on demand to avoid surplus animals from over-breeding.

While comparing treatment-groups, we will carry out as many conditions as possible in a single experiment to avoid repeated use of untreated/vehicle-treated/uninfected control groups.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

A mouse model causes the least overall harm compared to guinea pigs, rabbits and non-human primates, which experience more severe disease. Inbred mice are widely used and scientifically studied as models for human infectious diseases; their homogeneous genetic background reduces the number of animals required, compared with outbred animals.

Our experiments focus on early immune responses (innate immunity) and are more refined than longer and/or high-dose regimens that can cause longer-lasting harm and discomfort, and more severe disease outcomes. We will aim to use pre-defined time points that reduce pain and suffering, and use refined doses and administration schedule to reduce distress and harm.

Why can’t you use animals that are less sentient?

Adult mice are best suited for models of disease we will study. Insects (e.g. Drosophila) and less sentient vertebrates (e.g. zebrafish) do not possess the molecular components that drive disease- related processes in mammals, and are therefore not suitable for use in our studies. The mouse has a proven track-record of providing valuable insights on mammalian, including human, immune responses. Furthermore, there is a large collection of reagents and genetically altered animals in the field which will help our studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will keep up-to-date with literature to employ the latest transgene-inducing and imaging methods, including update our delivery methods, the substances and/or their formulations with the goal of reducing pain and discomfort to animals. We will aim to reduce the number of procedures on an animal by combining steps where possible, for instance, availability of slow-release formulations in the future could reduce the need for multiple injections over time. Where possible, will use sensitive read-outs that could enable us to collect key immune information at earlier time points and thereby reduce pain and suffering; for example, where possible we will use light- or fluorescence-based reporters.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow published guidelines issued by LASA, NC3Rs ARRIVE, and the RSPCA.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep ourselves updated with latest literature on optimisations and refinements of reagents, treatments, handling of mice etc. and follow any updates to ARRIVE and PREPARE guidelines when planning experiments. We will engage with the institutional 3R group, and keep up-to-date with and follow NC3R principles.

95. Nervous System Injury and Repair Strategies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

hypoxia-ischaemia, neonatal encephalopathy, CNS injury, PNS injury, oxidative stress

Animal types	Life stages
Mice	adult, pregnant, neonate, juvenile
Rats	adult, pregnant, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Blood and/or oxygen deprivation around the time of birth (birth asphyxia or neonatal hypoxia- ischaemia) affects 1-3 per 1000 live births in the developed world with rates up to 26 per 1000 in developing countries. The affected children either die, or the majority of survivors develop lifelong neurological disabilities such as cerebral palsy, mental retardation or epilepsy. Birth asphyxia can be caused by temporary disturbance of the blood supply to the uterus during pregnancy or through low oxygen levels in maternal blood thus affecting the neonatal brain. Currently, despite the quick development of

neonatal care, there is no cure for neonatal hypoxic-ischaemic brain injury and the only clinically approved treatment is through whole body or head cooling of the infant. This treatment is partially effective. Therefore, there is an urgent need for the development of new therapeutic strategies for neonatal hypoxic-ischaemic brain damage. This project aims to provide better understanding of the mechanisms behind neonatal hypoxic-ischaemic brain damage through breeding and use of transgenic mice and rats that are deficient or overexpressing molecules participating in cell death pathways involved in neonatal hypoxia-ischaemia. We aim to define the molecules which mediate inflammation, neuronal loss and behavioural deficits following neonatal hypoxia-ischaemia and to develop strategies to promote neuronal survival. Maternal infection also has a synergistic effect with neonatal hypoxia- ischaemia and contributes to the brain damage. In this project we aim to investigate the mechanisms behind this synergistic effect and to pharmacologically treat the pregnant mums in order to study and prevent the damage to the neonatal brain. We also aim to treat the fetuses with vectors and stem cells.

We aim to study the mechanisms of neuronal cell loss and tissue repair in adult brain hypoxia and investigate how development affects those mechanisms through comparison to hypoxia in the neonate, including the synergistic effect of infection.

We aim to identify factors mediating inflammation, loss of neuronal cells, fragmentation and disintegration of the axons, as well as axonal growth aiming to re-innervate the target muscles in models of neonatal and adult axonal damage, and compare these cellular changes and their mediators to those observed after local muscle paralysis, ischaemia and infection. The project aims to assess the effects of these factors on central nervous system (CNS) regeneration, particularly in spinal cord injury.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work? Background

Injury to the human brain around birth, during postnatal development or in the adult, frequently causes impairments in communication, movement and the ability to lead an independent life. The main sources of brain injury are trauma, infection and insufficient supply of oxygen and nutrients (hypoxia- ischaemia). The latter is either caused by global hypoxia (due to pregnancy complications affecting the fetus or cardiac arrest in the postnatal or adult life) or by obstruction of the of the blood supply to a part of the brain or spinal cord as in stroke. Neurological recovery after any form of brain and spinal cord injury is complicated by the low potential for neural repair as well as by the secondary brain damage.

Secondary brain damage is a common problem in stroke, brain trauma and hypoxia-ischaemia, and refers to increased neurological impairment following the initial insult to the

nervous tissue. It is often that the insult cannot be avoided and is accompanied by immediate cell death and tissue loss associated with primary damage. However, following restoration of the normal metabolism, a secondary more prevalent wave of delayed cell death and tissue loss occurs, which is related to secondary brain damage. During that stage inflammation plays a major part and contributes to the tissue loss.

However, an underlying infection can have a synergistic effect with an injury to the brain and cause collateral damage to non-infected tissue thus increasing the overall impairment as observed for example in neonatal hypoxia-ischaemia. The inflammatory response following neural trauma is strongly increased following application of an infectious stimulus.

The mechanisms causing secondary damage are poorly understood, but are mainly associated with low potential for repair and increase in energy demand, which causes molecular and morphological changes in the injured neurons, as well as activation of non-neuronal cells and recruitment of peripheral inflammatory cells to the site of injury.

This poor potential for repair after injury to the central neural tracts is partly due to exogenous inhibitors of regeneration, however many central neurons cannot repair and regenerate even if provided with an optimal environment. Failure to express essential molecules needed for regeneration seems to play a crucial role, but the precise minimal and optimal requirements are still only poorly understood. Even in peripheral nerve injury, where the potential for axonal regeneration is high, recovery in spatial maps and in fine perception necessary for precise and coordinated movements is very limited.

Work completed so far

The experiments carried out under the previous project licenses provided important information about the pathways and mechanisms involved in damage and repair of the injured nervous system. One branch of our research focuses on neonatal hypoxic-ischaemic (HI) insult and the mechanism underlying the subsequent brain damage. Data from our group demonstrated increase in acidosis during neonatal HI. We also examined the activation and deactivation of periventricular white matter phagocytic cells during postnatal mouse development and demonstrated the presence of highly active phagocytes in the neighbourhood of vulnerable white matter thus contributing or protecting against axonal damage in the fetus and premature neonate. Our work has also focused on identifying cell-type specific cytokines, transcription factors and signalling enzymes involved in neonatal HI brain damage, including Signal Transducer and Activator of Transcription 3 (STAT3) and extracellular signal-regulated kinase 2 (ERK2). We also looked at the effect of stem cells as treatment for neonatal HI brain damage. Recently, our research focused on the potential of natural compounds such as curcumin as treatment for neonatal HI brain damage.

We also looked at the synergistic effect between HI and systemic exposure to infection and demonstrated that genetic deletion of some pro-inflammatory cytokines can

completely abolishe the synergistic effect between infection and HI. We are currently investigating the cell-type specific role of different cytokines in neonatal HI and whether any of them alone or combined are responsible for the effects on the synergism of endotoxin and HI. Some of our studies looking at the synergistic effects of endotoxin and HI have focused on the role of post-translational modifications following HI, showing that their inhibition reduces neonatal HI brain damage thus suggesting this process as a potential target for pharmacological HI treatment . We are also blocked the alternative complement pathway through global deletion in neonatal HI demonstrated neuroprotection not only for HI, but also for infection- sensitised HI thus suggesting this blockade as a potential pharmacological target in neonatal HI brain injury.

Another branch of our work focuses on the molecular signals involved in neuronal regeneration following peripheral (PNS) and CNS injury. In these studies we investigate the role of transcription factors (STAT3, c-Jun), adhesion molecules and neurotrophins in neuronal survival and axonal regrowth. We confirmed that neuronal c-Jun is necessary for the promotion of regeneration, inflammation and cell death. We also studied the effects of c-Jun on injury-induced Schwann cell de- differentiation response and showed that c-Jun is essential to support motoneuron survival after nerve injury. We are also looking at the potential of natural compounds with anti-inflammatory and anti- oxidant properties, such as curcumin, as a way of provision of quicker and more successful regeneration.

Finally, we are also pursuing viral vectors as a method of tracing connections within the injured and regenerating nervous system, as well as delivering cytokines, trophic factors and other molecules beneficial to functional repair. Since many neurons stop dividing during late fetal or postnatal development, they cannot be replaced by the organism if neuronal loss occurs as the result of failure to implement early neural rescue therapy. Replacement therapies, using normal or genetically modified neural stem cells, therefore hold the key to treating patients with neural loss when the time window for early intervention has passed

What outputs do you think you will see at the end of this project?

This work aims at reduction of the incidence, severity or prevention of neonatal hypoxic-ischaemic brain damage thus saving babies' lives and reducing the socio-economic burden to the affected individuals, families and healthcare system. This project also aims at helping people with brain damage through better understanding and development of potential treatment for patients suffering from bacterial meningitis, head trauma, hypoxia and stroke and improvement of their recovery of mental, sensory and motor functions.

Who or what will benefit from these outputs, and how?

The project will provide insight into the pathways involved in injury to the nervous system and thus opportunities for the development of treatment for people with brain damage.

The proposed project will provide knowledge on the involvement of different transcription factors and their post-translational modifications, as well as oxidative stress, infection and inflammation in cell death pathways triggered by neonatal hypoxia-ischaemia, and adult and neonatal CNS and PNS damage due to bacterial meningitis, head trauma and stroke. The work on the proposed project will allow use of these proteins as targets for inhibition and prevention of neonatal and adult CNS and PNS damage with potential future application in the clinical practice.

Due to the lifelong nature of the disabilities caused by neonatal HI, they impose an emotional and financial burden not just for the affected individuals and their families, but also for the society and healthcare system.

The results from the current project will benefit the scientific society increasing the knowledge on the involvement of different transcription factors, infection and inflammation and oxidative stress in neonatal and adult brain damage, and will provide potential strategies for targeting and treatment of neonatal and adult CNS and PNS damage. Work described in the proposed project will provide numerous benefits in terms of uncovering the molecular basis of brain disease, developing and testing new forms of treatment and giving a rational basis for intervention in human patients.

So far the only clinically approved treatment for neonatal HI is whole body or head cooling, however its success rate is limited and 40% of the treated children still suffer from neurological disabilities. This urges the need for additional or alternative therapies to be used either alone or in combination with cooling to reduce neonatal and perinatal brain damage. Reduction of increased transcription factors or inhibition of their post-translational modifications, inhibition of oxidative stress, maintenance of the balance of pro- and anti-inflammatory cytokines following neonatal HI and adult and neonatal CNS and PNS damage alone or in combination with cooling can prove beneficial and following testing in rodents, can be used in translational models and potentially clinical trials.

How will you look to maximise the outputs of this work?

We will be looking into establishing collaborations with academics, as well as industry. All the data produced in the project will be published and presented at conferences. We will also involve members of the public through participation in In2Science and Nuffield programmes providing university internship placements for young people from minority backgrounds.

Species and numbers of animals expected to be used

- Mice: 16100
- Rats: 700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Experimental animals will be limited to mice and rats – mice because of the availability of many genetically modified strains, and rats because it allows us to perform surgically elaborate experiments in a larger, docile rodent, with – for a mammal - comparatively moderate level of brain development. The neonatal mouse and rat brain developmentally corresponds to late preterm/term human neonate, thus providing clinical relevance to the outcomes of neonatal hypoxia-ischaemia.

Typically, what will be done to an animal used in your project?

After being bred in Protocol 1 the animal will undergo anaesthesia and will be subjected to surgery (blood vessel occlusion or nerve crush or cut). The animals are pre- or post-treated with different compounds and will remain on study for up to 6 months when they might be used for behavioural assessment and/or subjected to induction of general anaesthesia and injection of tracer 2-3 days before planned killing. The animals are killed either by Schedule 1 or by terminal barbiturate anaesthesia and perfusion with fixative to allow better histological specimen.

What are the expected impacts and/or adverse effects for the animals during your project?

Ear notching should involve only slight and transient pain, and no healing problems. Pain from tail tipping will be controlled by local or general anaesthestic, and in any bleeding after tail-tipping or blood sampling by local pressure. Excessive blood sampling may cause anaemia or haemodilution, so the amount withdrawn will not exceed 15% total blood volume in any 4-week period.

Animals who are immunodeficient lack differentiated T- and B-cells. Thus infections are likely to occur. In animals used in models of motorneuron diseases, paralysis might rarely occur at earlier stage.

Mild cardiorespiratory depression due to anaesthesia and surgery is rare; postoperative pain, post- operative infection (inadvertent), stress, abortion, agranulocytosis. Minimal distress associated with behavioural tasks. No anticipated effects on the dam due to chronic exposure to a hypoxic air mixture (Baud et al., Brain Pathol. 14:1, 2004). Mild stress likely. Animals are likely to become distressed during behavioural tasks. Incidence of other adverse effects is low.

Injection of substances could rarely result in distress. Injection of LPS could very rarely result in sepsis.

Following a Caesarian section the pups will be fostered, but rarely they might be rejected by the foster mum. Following normal delivery, the pups rarely might get rejected by the mum.

Very rarely some animals (<1%) who will be anaesthetised and/or intracerebrally injected, or animals re-anaesthetised for nuclear resonance scanning, might die.

Loss of animals due to anaesthesia or surgery is rare; postoperative pain, post-operative infection (very low), agranulocytosis (very low). Minimal distress associated with behavioural tasks is very low. Sometimes hypoxic ischemic insult following surgery in some strains of mice may result in loss of up to 15% of animals. Some stress is likely during behavioural tests. If an animal struggles in any of the behavioural tests it will be immediately removed from the experiment. Injection of substances could rarely result in distress. Injection of LPS could very rarely result in sepsis. Following HI the pups very rarely might be rejected by the mums. Very rarely some animals who will be anaesthetised and/or intracerebrally injected, or animals re-anaesthetised for nuclear resonance scanning, might die.

Incidence of other adverse effects is low.

- We are seeking to reduce the post-operative mortality rate through:

• Avoidance of use of first litters (those are known to be weaker, runty and often neglected by first time mums)

• Our experience shows that offspring of breeders generated through in house breeding seem to tolerate the insult better and show less abnormalities than those obtained from breeding of commercially purchased animals.

• Currently the device we use allows hypoxia of a group of animals without individual access, and opening it during the experiment would result in re-oxygenation of the rest of the group and would obscure the results. This can explain the postoperative lethality in some strains of mice. On the other hand single animal exposure to hypoxia one at a time is not feasible and would not provide equal exposure to the same conditions of the experimental animals, thus introducing an additional variable and error in the experiment. Optimisation of the device providing hypoxia allowing single animal exposure would allow us to terminate the experiment for each animal as soon as we observe abnormal behaviour associated with suffering and pain. Such device will be put in use as soon as it is available.

• We intend to use therapeutic hypothermia in neonatal mice and are aiming to replace the currently used rectal thermal probes with pacifier thermometer, infrared thermometer, external thoracic thermal probe or a thermal camera, hence avoiding additional distress to the animals through continuous exposure to anaesthesia and invasive procedures, and complying with the 3Rs.

Following neonatal axotomy the pups very rarely might be rejected by the mums. Partial paralysis as a consequence of axotomy is expected, but that is temporary and reversible after 10 days following surgery. In some mouse strains the paralysis might extend beyond 10 days but would not affect behaviour, feeding and motor performance.

Excitotoxic agents can cause convulsions and fits, therefore death is also expected. With the dose to be used in the experiments we would not expect epileptic fits, however the incidence of such is very low (<1%). Incidence of other adverse effects is low.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect all the animals in protocol 1 to have mild severity. We expect 70% of the animals in protocols 2 and 3 to have moderate severity and the rest to have sub-threshold. The animals in protocols 4-6: we expect 60% for the animals in each of the protocols to show moderate severity and the rest 40% to show sub-threshold severity. We expect 50% of the animals in protocol 7 to experience moderate severity and the rest 50% to show sub-threshold.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Work in cell culture has played a pivotal role in many aspects of the projected work, in the identification of neurotrophic substances, excitotoxicity, and the intracellular cell death and differentiation pathways. However, in vitro studies deal with simplified systems, and cannot in the final analysis predict, whether a specific molecule has a detrimental, beneficial effect OR, just as importantly, simply no effect in the complex organism. To answer these specific questions, one cannot avoid doing these experiments in vivo.

Which non-animal alternatives did you consider for use in this project?

Cell cultures, computer modelling

Why were they not suitable?

In vitro studies provide a very simplified isolated system for testing, which can provide some information about the mechanisms underlying neuronal injury and repair. However, these mechanisms are to a great extent associated with intracellular communications which cannot be provided through in vitro testing, but only in the in vivo experiments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Sufficient predictive power to observe a significant effect or its absence is a key requirement in planning animal experimental work. This applies for all experimental stimuli, in our case a deleted gene, applied neurotrophin, or a specific form of injury. Work started with insufficient number of animals only entails animal suffering without producing significant insights into biology of the disease.

Moreover, each animal is individual and most biological responses show strong variability, even in inbred animals. Thus, previous work in our group showed a relative standard deviation (SD/mean) of 40-80% for lymphocyte recruitment to the injured brain and 25-50% for neuronal cell death. Based on these numbers, we will need a group size of approximately 16 animals per group to be able to identify a statistically significant, 50% reduction. In a comparison between 2 groups of animals (e.g. treated v untreated), we will need 20 animals per test. Taking into account license duration (5 years), this will amount to 10 experiments per protocol and year. This is not unreasonable in view of the number of mutant mice or therapeutic substances in mice and rats that will need to be tested in this programme. Increasing this number has the inherent danger of finding weak but statistically significant effects of questionable clinical relevance. We are also aware that some biological responses show much lower level of variability (10-20%). If this is the case in any of the responses investigated in this programme, we will reduce the number accordingly, down to 4 per group, the lowest number required for a statistically valid assessment. In our experience, such a flexible approach will reduce the overall number of animals, decrease economic cost and minimize animal suffering while still permitting a valid statistical comparison.

In addition to the above, paring down on unneeded group size, we will, whenever possible try to avoid duplication of control groups, using box analysis and ANOVA testing several experimental groups against same group of controls.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experimental Design Assistant (NC3Rs), ARRIVE guidelines, using data from past experiments as ground for generation of pilot studies, http://powerandsamplesize.com/Calculators/Compare-2- Means/2-Sample-Equality

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Efficient breeding, cell culturing to replace the use of animals in toxicology essays, and sharing of tissue

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Breeding of wild type and genetically altered animals with mild and moderate severity, maternal surgery/hypoxia and treatment of the offspring, neonatal hypoxia-ischaemia using a modified version of the Rice-Vannucci model, neonatal and adult axonal cut or crush, or local muscle paralysis, adult hypoxia-ischaemia as a model for blood vessel blocking events.

Why can’t you use animals that are less sentient?

Experimental animals will be limited to mice and rats – mice because of the availability of many genetically modified strains, and rats because it allows us to perform surgically elaborate experiments in a larger, docile rodent, with – for a mammal - comparatively moderate level of brain development.

The resemblance between the rodent and human nervous system is well described. Even though there are also similarities with earlier developmental life forms (i.e. C. elegans), those can be suitable for some aspects of cell response, but due to the developmental differences would not provide data which can be incorporated for use in large animal models or clinical trials.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal experiments can lead to considerable stress and it is essential to minimize suffering. In the current programme this will be achieved by limiting extensive, major

surgery (trauma, blood vessel occlusion) to one event. All additional, short invasive procedures will be performed under brief, general anaesthesia. Experiments with animals showing obvious signs of distress and pain (immobility, unkempt fur, no passing of urine and faeces), rapid loss of more than 15% of body weight, will be terminated prematurely. As infection and sepsis have deteriorating effect in neurological diseases, it is important for us to explore their effect in our models. However, in order to control infection and potential lethal outcome, we will be using sterile gram-positive or gram-negative bacterial components so we can mimic those complications under controlled conditions.

Experimental animals will be limited to mice and rats – mice because of the availability of many genetically modified strains, and rats because it allows us to perform surgically elaborate experiments in a larger, docile rodent, with – for a mammal - comparatively moderate level of brain development

Anaesthesia will be achieved by using always the most refined method for the scientific purpose. In neonatal mice from birth will be subjected to inhaled gas or injectable anaesthesia will be used. There are suggestions that pain may be experienced on recovery. In adult animals is assessed through changes in behavior, while in neonates pain is assessed through changes in skin and stomach colour, and rejection by the mum. In our models inflammation is a crucial factor in the trigger of injury, therefore we are unable to use NSAIDS as they would interfere with the outcome. We will try alternative pre-operative analgesia (i.e. opioids, local anaesthesia) and if the effects are detrimental, then we would need to use post-operative pain relief.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs and ARRIVE guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through participation in NC3Rs webinars and close communication with Biological Services and the HO

96. Understanding Initiation Events for Breast Cancer and How Pregnancy may Influence These

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

breast, cancer, initiation events, pregnancy protection

Animal types	Life stages
Mice	juvenile, adult, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand the early initiation events that lead to breast cancer, and how pregnancy may influence either early events or downstream events.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In the UK alone over 50,000 women per year are diagnosed with breast cancer, with the majority of these occurring in women post menopause. Research into breast cancer often centres around the detection and treatment of disease, and although this is hugely valuable, understanding initiation events could go a long way to prevention over treatment. Thus far there is limited knowledge surrounding how cancers are initiated. Some, such as colon cancer, come with consistent changes in the genome which could be causative. Breast cancers, despite sharing many features across patients, allowing for subtype generation, have few known shared features which would be suggestive of initiation. BRCA mutations, being one such gene, are only present in 3% of breast cancers, so although highly useful in predicting future breast cancer events, it is clear there is more to discover.

What outputs do you think you will see at the end of this project?

This project will provide detailed information including, gene expression (RNA) and DNA sequencing, epigenetic (the modifications to genes that cannot be seen from standard RNA and DNA sequencing), and environmental cell modifications, regarding the changes that a mammary gland undergoes in the development of mammary carcinoma, and how these may differ before and after pregnancy. We hope to produce peer reviewed publications and provide a good groundwork for on-going research in this area with the development of potentially new (or more developed) and useful models, and an enriched data set that has benefited from up-to-date and advanced methods.

Who or what will benefit from these outputs, and how?

In the short term those working in the field may benefit from any changes to the current experimental designs that are used to study the effects of pregnancy on breast cancer development, particularly those that shorten the time to obtain results. The research field will also benefit from any data that is made available, this will be of interest not only in those working on pregnancy protection, but also on breast cancer in general.

In the long term we hope that the general population will benefit from a treatment that can act as a preventative rather than a cure.

How will you look to maximise the outputs of this work?

We will be open to collaborate with other laboratories that have interesting models, data, or questions where our methods can be applied. Moreover, all the knowledge and expertise generated with this project will be spread through talks, conferences, and publications.

Species and numbers of animals expected to be used

- Mice: 2272 mice

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the least sentient, and most understood (in terms of gene expression and gene modification and cell labelling) animals that represent mammalian biology. They allow us to recapitulate human disease in a way that other, less sentient, animals cannot do. We have many years of experience working on breast cancer in this animal model and have developed tools and strategies that are most suited to mice. We will work with both adult mice and juvenile mice. Adult mice are more tolerant of the methods we wish to use, and they are more developed in terms of their mammary gland / breast tissue system (similar to a human who develops breast cancer would be), whereas juvenile mice (<4 weeks) will be used where we wish to create a newly modified mammary gland. In these cases, their gland will be less developed, thus for transplantation assays this makes for efficient clearance of the mammary gland. Egg/embryo and neonate are included in the event they may be affected (via the ovaries) by the mum having received carcinogen treatment prior to undergoing a pregnancy.

Typically, what will be done to an animal used in your project?

- Typically, an animal will be anesthetized, and a vaginal swab may be taken prior to receiving treatment (to ensure results are not affected by different stages of oestrus cycle). At a designated time the animal will be exposed to a solution containing a cancer causing agent (carcinogen), potentially as an intraductal (via the nipple) injection (termed MIND) under anaesthetic or in the form of oral gavage (direct application through the mouth) (once a week for up to 6 weeks). Mice will be killed at designated timepoints after carcinogen exposure and their mammary glands removed. Some groups of mice from this treatment may be allowed to develop small tumours. In cases where we are looking at the early mammary gland response to carcinogens, mice may not even progress to develop small tumours before being humanely killed

- Alternatively, a mouse may be anesthetized and then have its mammary gland removed. The mouse then may be humanely killed or, for transplantation assays will be injected with mammary gland stem cells which will result in the growth of a new mammary gland with the same genetic make-up as the implanted stem cells. That same mouse may then be exposed to a carcinogen (as above) and some groups of mice may develop small tumours. Some mice may undergo a pregnancy at variable stages throughout these procedures.

- In some cases where we will generate a transgenic mammary gland via an electroporation technique (as opposed to transplantation), mice may be anesthetized and undergo an injection of virus into the mammary gland (via MIND or applied to the fat pad directly), followed immediately by a small electrical current being applied to the exposed gland using tweezer electrodes. That same mouse may then be exposed to a carcinogen (as above) and some groups of mice may develop small tumours. Some mice may undergo a pregnancy at variable stages throughout these procedures.

- A small number of mice (those not having undergone any of the afore mentioned procedures) will go through forced involution, in that pups will be removed within 24 hours of birth, resulting in the mammary gland returning to a non-breast feeding state (as would typically happen once pups have been fully weened). A subset of these post pregnant mice will be kept alive beyond 12 months of age (not exceeding 15 months).

What are the expected impacts and/or adverse effects for the animals during your project?

The mouse may feel some discomfort in the mammary gland/ nipple location for all procedures, but we anticipate this to be transient and reduced with analgesics if required. The mouse may develop small (~0.5cm or less) tumours but the location and size should not cause significant discomfort.

Mice undergoing forced involution may experience some distress with the removal of pups. This may be mitigated by allowing killed pups to be placed back into the cage for a short period of time to allow the mother to come to terms with their loss, or by the addition of 'toys' to provide some distraction.

Mice undergoing forced involution may experience mastitis (although this is unlikely), we will observe these mice carefully to detect this early.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For animals where we are maintaining the tumour growth in vivo the severity would not exceed moderate. For recipient mice developing a new mammary gland from donated stem cells, the severity will also be moderate. For animals which we use for whole organ / or part organ mammary gland culture, or as stem cell donor mice for transplantation assays, or where we will look at very early cell response in the gland to a carcinogen, the severity would be mild. The proportions may somewhat depend on the success of each experiment, but we anticipate a 50% ratio between moderate and mild.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Breast cancer can only truly be studied on animals as it is a complex disease that involves many cell types and many interactions. To combine this with pregnancy makes it impossible to use a cell line/ culture system independent of animals.

Which non-animal alternatives did you consider for use in this project?

We will work hard in this project to develop the use of the mammary gland whole organ / part organ culture assay to address some questions. Although this does not eliminate animals completely, it does remove their conscious harm and could allow us to obtain preliminary data to optimise our use of in vivo models. By using a transplantation model for the generation of genetically modified mammary glands we reduce the need to generate large colonies of GM mice.

Why were they not suitable?

We hope our in vitro assays will generate useful data. Regardless of success here, these assay conditions are not suitable for answering some of the more complex questions that require a whole body system.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated this number of animals based on the number of conditions that we plan to test, i.e., the number of different treatment options, and the number of animals that are in each group to ensure we have significant confidence in our result. We have also included the possibility that unforeseen outcomes would occur, requiring us to use additional mice to ensure we have significant confidence in our result.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have followed the best available guidelines on experimental design, including these listed on the NC3R website. In addition, we screened the existing literature to inform ourselves on the best standards currently applied in terms of sample size determination.

Wherever possible, we have attempted to perform multiple measurements from the same dataset (applying the appropriate corrections) and to compare multiple treatments to the same control, reducing the number of control animals required. In addition, where possible we will use independent regions of the mammary gland - both left and right glands, and also distinct regions within the same gland, to compare multiple experiments, and thereby reduce the number of animals required for our experiments. We will also maximize the number of host mice that can receive tissue from a single donor mouse. We will also maximize the number of host mice that can receive tissue from a single donor mouse.

We based our sample size calculations on estimates of effect size and variability from previous data of our laboratory. However, this is just an initial estimate, and we fully expect that our understanding of the data will increase over the project. As this happens, we will refine our sample size calculations and either use less animals, if possible, without reducing scientific significance, or seek permission to enrol more animals in the study if we realize that a higher number of replicates is needed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Any mouse colonies will also be maintained using efficient breeding.

We will use both the left and right mammary glands for the majority of our experiments. We will also investigate the possibility of testing multiple gene modifications even within the same gland. We will also maximize the number of host mice that can receive tissue from a single donor mouse.

We will carry out pilot studies, prior to data collecting studies, to ensure we have developed the techniques to the best of our abilities and that they reflect the desired outcome in terms of gene expression/ gland development etc.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We intend to use mouse model systems during this project. Part of our project will be to develop optimal cancer inducing models for the breast. Much of the methods we wish to use are via injection through the nipple into the mammary duct of the mouse, which is considered more representative to human disease (as ~80% of human breast cancers diagnosed are considered to originate from the duct), and is also a far less invasive method for the mouse than standard mammary gland injections (via surgical procedure). Injection into the nipple of the solution containing the cancer inducing agent (carcinogen), would be a refinement over the standard procedure (administration via the mouth) as this reduces the number of body parts exposed to the carcinogen, and is a less distressing technique for the animal (given the animal will be anaesthetised during the nipple injection, where they are not when given the treatment via the mouth).

Why can’t you use animals that are less sentient?

In some instances, we intend to use animals that have been terminally anaesthetised. For instances where we require a transformed duct (either as an early or later stage cancer), we need to make use of the entire body system, and all the different cell types that come with this. In other instances, we may require an extended latency period before we see a cell effect after the cancer inducing treatment, so to maintain the cells alive and in their natural environment we must do this in a living mouse. To look for any effects from pregnancy, we require a mature animal that will represent all the different stages of pregnancy, pre and post.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are proposing to induce cancers via injection of cancer-causing agents directly into the nipple of the mouse. This should cause minimal discomfort for the mouse with no chance of cancer cells expanding between the internal skin layer and the mammary gland and no ulcerations because of this. Typical administration of carcinogens, such as the one we are proposing to use, involved oral application. This inevitably effects more than just the mammary duct and cancers are known to develop in many other organs. We wish to refine this by injecting directly into the mammary ductal via the nipple, thereby reducing the effects on other organs. We are also planning to use a culture system, where possible, to treat or observe a removed [from the mouse] mammary gland and its response to cancer.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our institute has a series of guidelines regulating many of the procedures routinely done to animals, for instance how often an animal can receive an injection, where, and how much,

or how often it can give a blood sample. We will follow all of these, as well as several other standard operating procedures that were developed by a team of specialists at our institute for the explicit purpose of minimizing animal suffering and are periodically updated. While designing experiments we will follow a series of guidelines existing in the literature to design and report our experiments, and will continually consult with an on-site statistician to ensure that we're using as few animals as possible for our study.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institute routinely circulate advances in the 3Rs and we will always seek to identify ways these can be incorporated in our project, while ensuring they do not affect the (statistical/ biologically relevant) consistency of our data collection

97. Behavioural and Neurochemical Mechanisms of Innate and Learned Resilience in Psychiatric Disorders

Project duration

5 years 0 months

Project purpose

(a) Basic research

Key words

Anxiety, Fear, Behavioural Neuroscience, Memory, Brain

Animal types	Life stages
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The proposed work programme will address pertinent gaps in our knowledge regarding how an experience is encoded and persists as a memory that impacts long term behaviour. We aim to elucidate what behavioural interventions and neurochemical mechanisms can boost innate or acquired resilience against anxiety using rodent models.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The function of memory is not to simply look back on experience, but to facilitate adaptive behaviour in light of new challenges. Despite some progress, our fundamental

understanding of how memory is encoded in the brain and how memories can influence subsequent behaviour is still limited. Building our knowledge of how memory 'works' is a priority for discovery neuroscience of this generation. The proposed work programme will specifically examine how memory of an emotionally aversive experience can influence future behaviour, such as vigilance for potential danger. In terms of translation into benefits for Human health, this work will refine rodent models of anxiety disorders and identify mechanisms underlying symptoms that are found across a number of psychiatric disorders including PTSD, Depression, OCD and Phobias. In this programme of work, we will develop our knowledge of what factors (innate or learned) influence resilience to aversive experience. Additionally, as we actively collaborate with pharmaceutical partners in industry, there is real potential that refined models and novel knowledge of the mechanisms underlying resilience could be used for testing of innovative drug therapeutics in the longer term.

What outputs do you think you will see at the end of this project?

By comparing the underlying biological mechanisms that control responses to potential danger that are innate (biologically 'hard-wired') with those that are acquired by experience (remembered), this project will advance our understanding on why hypervigilant responses are difficult to diminish, i.e. resistant to therapy. This is a question of fundamental importance for understanding how memory updating works.

This programme of work will refine the measures used to analyse memory in rodents. We will develop tasks to be able to better detect and measure behaviours akin to symptoms seen in Humans, such as hypervigilance for danger signals. These protocols will be published in wide reaching scientific journals and our computational scripts and research data will be made open access for other researchers to benefit from this information.

Moreover, the findings will have direct pre-clinical relevance for treatment of disorders like anxiety, Phobias and PTSD given we will investigate the analogous neural circuitry that is thought to be disrupted in these conditions. We already collaborate with pharmaceutical industry partners, and we actively seek clinical collaborations in the interests of both enhancing awareness of our basic findings, and refining our research questions based upon clinician and patient experience.

For all objectives, we regularly communicate our data at scientific meetings (conferences, minimum around 3 a year) and publish in peer reviewed journals (at least once a year). Therefore, we would expect at least five original articles to result from this project (original data and theoretical).

Who or what will benefit from these outputs, and how?

In the immediate term, our research group will directly benefit from data concerning the validity of the models as the data is analysed, as these will be used to inform future studies (either continuing with the same model, or investigating alternatives where models are not

validated). In the first two years, we will develop new protocols to screen for individual differences in hypervigilance to potential danger (under conditions of safety), building on our knowledge of experimental psychology and behavioural neuroscience. Scientists in the field of memory research and/or anxiety research will benefit from the fundamental knowledge of how memory works. We will use state of the art techniques to target manipulations to specific neural pathways to understand how specific behaviours are generated. This programme of work will reveal mechanisms underlying learning from aversive experiences that will address outstanding scientific questions. The publication and dissemination of the data should occur within the timeframe of the licence, or shortly afterwards, which will support the field and other groups in their research efforts.

In the long term, these protocols will be investigated pharmacologically using drugs that are deemed to reduce anxiety in Humans to understand how those drugs work and identify potential new targets.

Psychiatric disorders remain a significant challenge for translation. Moreover, as we actively collaborate with industry pharmaceutical partners, there is real potential that a valid model and novel knowledge of mechanisms could be used for testing of novel therapeutics in the longer term.

How will you look to maximise the outputs of this work?

The proposed research programme will bolster and develop our collaborative projects. We actively collaborate with other groups working on mechanisms of memory and have also collaborated with animal welfare researchers to examine biomarkers for welfare/affective state. Nationally, we have active collaborations with groups studying models of psychiatric disorders such as OCD and internationally with industry partners on the development of translational rodent models for psychiatric disorders.

Our primary data papers are published in Open Access format (i.e. free-to-view immediately on publication). Our commitment to Open Access and move to open data maximises the benefits of our research to the academic community, which includes both basic scientists and clinicians. Furthermore, we have a proven track record in publication of negative findings and striving against the positive findings publication biases that exist in the literature.

We also are active in public dissemination of our work. I have contributed to events at Science Festivals. I have also presented internationally at outreach events like “TedX” in Cyprus. I have given many online media (radio, podcast, vlog) contributions to disseminate the knowledge gained by similar research projects.

Species and numbers of animals expected to be used

- Rats: Adult Rats total 2580

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To further our understanding of how memory works and how threats are detected at the neurobiological level we have chosen the adult rat as the most suitable animal model for this project. We cannot use a ‘lower’ species of animal at present, due to a need for behavioural complexity that necessitates an ‘intelligent’ animal, with complex social learning behaviours. The rat is a superior model to the mouse for this programme of work given that one readout we are particularly interested in is ultrasonic vocalisation calls. Mice are documented to produce less rich ultrasonic vocalisations, which is argued to reflect that they have evolved a different repertoire of social behaviours and live under different social hierarchies than rats. Moreover, it is generally accepted that the brain cortical structures involved in learning and memory are more tractable to study in the adult rat than mouse.

We have chosen adults, as their behavioural repertoire is more developed than juveniles, and in terms of biological mechanisms there is a developmental regulation of synaptic proteins in the brain areas we are interested in studying. The majority of the experiments proposed will require testing of males as there is evidence that the behavioural responses of female rats to danger cues are different to male rats and cannot be easily compared. Nonetheless, we are fully aware that psychiatric disorders of anxiety are more commonly diagnosed in women (though this is likely both due to biological and societal factors). Therefore, a subset of work will be carried out on defined predictions (such as dominance testing and screening for vigilance responses) to see whether the protocols can apply to female rats.

Typically, what will be done to an animal used in your project?

In general, our experiments are of a relatively short duration of approximately 2-3 months maximum. A typical rat in an experiment will be housed in a social group upon arrival to the unit and acclimatised for a week. With the exceptions of after a surgical manipulation, rats will be housed in social groups to allow social interaction and with environmental enrichment (e.g. cardboard tubes and chewing blocks) present throughout the duration of the experiments.

Their dominance level within the social group will be determined (see below). They will then be behaviourally examined for characteristics that relate to potential danger response behaviour (e.g. avoidance of lit areas in mazes). Then using aversively driven tasks (pavlovian conditioning), the demonstration of species typical responses to learned (associative) and/or innate (agoraphobia, predator odour) potential danger cues such as freezing behaviour and ultrasonic vocalisations (USVs) will be measured. Then

interventions (behavioural, pharmacological, neural circuit manipulation) will be performed on the rat to understand how that memory was formed and to enhance resilience to subsequent threat responses.

In order to study resilience to potential threat (Objective 1 and 2) this programme of work requires the use of moderate aversive stimuli such as inescapable electric footshock. The majority of animals (<80%) will experience this. Some animals will not be directly exposed to footshocks. These exceptions are for vicarious conditioning experiments, where rats observe a conditioned cage-mate and do not experience any aversive stimulus directly (but learn through observation of the other rat), and Control groups where rats are exposed only to the associated cues (context, sounds, smells) in absence of any aversive stimulus (to measure the specificity of learning).

The typical response of a rodent to an electric footshock is to jump and vocalise for less than a second or so, and then resume normal behaviour. The level of shock we use produces no physical lasting harm to the animal, however, is enough to lead to an aversive memory being formed, which we later measure under conditions of safety. Few aversive stimuli are typically needed to effectively train the animal (<5 presentation of footshock for robust learning). These exposures will be limited to maximum of 4 in any test session and not more than 10 in the lifetime of the animal. The subsequent responses of the rat, such as freezing and USVs (which we will measure), are specifically generated only when the memory recall is tested (and not in the home cage or other environments).

In order to compare learned responses with innate (untrained) responses some rats (<40%) will undergo exposure to innocuous mild stressors such as:

- Derivatives of predator odour: Typical response = avoidance, USVs, freezing
- Elevated mazes or open arenas: Typical response = avoidance, USVs, thigmotaxis (keeping to the wall)
- Observation of another rats' responses (vicarious conditioning): Typical response = USVs, freezing
- Playback of ultrasonic alarm calls of a previously conditioned rat: Typical response = avoidance, USVs, freezing.

We will also study the influence of social hierarchies on defensive behaviour (particularly on vicarious learning and USV production) using tasks that can elucidate the hierarchy based on appetitive resource competition. This appetitive measure of competition does not trigger notable aggressive behaviour during the session nor afterwards on return to home cage, and involves brief (5 min) access to a highly palatable food resource, where rats compete for physical access to a spout to drink, and this can be carried out without food restriction (refinement we carried out from previous licence PA9FBFA9F) using a very palatable drink.

A central goal in understanding brain function is to establish causal relationships between specific cell populations and behavioural outputs to ensure construct validity for relevance in Humans. To test a specific requirement of discrete neurochemical systems in a particular brain region of interest, the rats may undergo a surgical procedure (<25%) performed under general anaesthesia to be implanted with chronic, indwelling guide cannulae or administered viruses to target specific brain regions. Rat will be given pain killers during recovery and typically resume normal behaviour, (active locomotive behaviour, appetite, and responsiveness) within a few hours following surgery. In those animals fitted with cannulae, pharmacological substances may be administered locally to modulate brain activity to test whether our developed tasks have the potential to screen for drugs that are effective in treating Humans. The dose and route of administration will be based on careful review of best practice in the existing literature. At the end of the study the animals will be anaesthetised and killed, and brain tissue collected for further analysis of the mechanisms underlying memory.

What are the expected impacts and/or adverse effects for the animals during your project?

We will design all experiments in consultation with the LASA guiding principle documents, particularly for Behavioural Laboratory Animal Science (2013). We always prepare ‘Pre-Study Briefings’ prior to any experiment where the 3Rs are also considered to minimise any predicted harm to the animals and these will be discussed at team meetings. We maintain detailed records in laboratory notebooks alongside electronic shared cloud storage to maintain all details relevant to the particular procedure (e.g. ambient light intensity, animal weight, food consumption) will be noted as a matter of routine.

For most rats the experiments will be exposure to brief stressors such as injection or brief foot-shocks. We administer injectables using refined procedures to avoid any stress of restraint and the number of administrations will not exceed 20 in the experience of the animal. The level and duration of foot- shocks we routinely use for sufficient associative learning are comparatively weaker than those used often in the rat literature and do not cause any chronic change in general behaviour of the rodent. The acute affect at the point of administration is brief jumping and/or vocalisation for a few seconds. Rats are regularly monitored throughout the sessions in real-time by CCTV footage. The experimenters have years of experience using these procedures and we have never observed tissue damage in the animals, nor have we seen any indications of distress other than the specific behaviour of ‘conditioned freezing’ that animals display on re-exposure to the shock-associated cues or context (under conditions of safety). Furthermore, this ‘conditioned freezing’ behaviour can be quickly extinguished, leading us to believe that despite the use of inescapable shock, this procedure is refined in terms of animal suffering and offers clear parametric control.

Surgery is conducted in accordance with aseptic surgical guidelines, and all Personal Licence Holders conducting surgery receive training specifically on aseptic technique.

Surgery is conducted by a Personal Licence Holder who is trained in aseptic surgery. Any surgeries will be performed under anaesthesia and analgesia, and post-operative analgesia will be administered during the first three days of recovery, which will last at least seven days before any other procedures. Animals are expected to make a rapid and unremarkable recovery from surgical procedures. Analgesic agents will be administered as required. No post-operative complications are anticipated but in the event of any post-operative complications, the opinion of the Named Veterinary Surgeon will be sought to see if such complications can be remedied promptly and successfully using no more than minor interventions. In the case of wound dehiscence, uninfected wounds may be re-closed on one occasion within 48 hours of the initial surgery. Rats with 'head caps' after cannulation (<25%) will need to be initially singly housed to prevent any damage from cage mates to the head cap. In rare cases (<5%) the head cap can become dislodged. If the animal can continue in the experiment as there is no sign of distress, nor clinical signs of ill-health and no longer requires the implant (e.g. it has completed the stage of the experiment for which this is required) then, following consultation with the NVS, the animal may be allowed to continue in the experiment. The majority of animals are not expected to show any persistent clinical signs after one week.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Overall, 93% of the animals are predicted to experience a moderate severity in this project as the behavioural tasks used are aversively driven for the key experience of the animals to model aversive experience learning. A subset of (<7%) will be tested using appetitive (palatable reward) or mild tasks (predator odour) for characterisation and comparison of the mechanisms of appetitive versus aversive learning and innate responses. The vast majority of the animals will undergo behavioural testing of an acute nature (>90%, associative learning or innate threat detection). To validate task translation and investigate neurobiological mechanisms underlying experience-driven behaviour a proportion will receive systemic pharmacological manipulations (~38%). Those that undergo surgical procedures (<25%) would be classed as a moderate severity procedure for the implantation of cannulae or viral transfection for local brain region manipulation.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project aims to measure behavioural responses to threat cues to understand the mechanisms by which the brain coordinates vigilance as a cognitive function. It is not possible to replace this programme of work using an in vitro or ex vivo model as a key outcome measure depends on measuring behavioural responses in the intact rodent, which we consider the least sentient creature for comparison to humans in these outcomes.

Which non-animal alternatives did you consider for use in this project?

In silico Models (Computational), Ex vivo (Brain Slice), and In vitro (Cell Culture) Models.

Why were they not suitable?

These non-vivo models are currently not viable to address the behavioural predictions of this project. Nonetheless the data collected can be subsequently fed back into models to make predictions on driving factors for subsequent work. For example, work on the parameters of how much new information is needed for the animal to update their memory has fed into theoretical models into how learning works.

For future work the behavioural data we collect would provide a useful basis for computational modelling to make some predictions on which factors to focus on, but the observation and measure of real behaviour is necessary first. We are well placed to establish collaborations and networks, to take this forward in the long-term following the completion of this programme of work.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The specific numbers of animals estimated for each protocol is based off predictions given our experimental design, including replication and randomisation. Given our track record and projections for future funding and research team size these numbers reflect our predictions to address the programme aims. Estimates of groups sizes are more difficult for novel interventions, so in these cases we will use pilot experiments and power calculations based on an estimated desired effect size. The vast majority of the experimental approaches in this programme of work have been done before on other licences, and as such we draw on the returns for those licences to bolster our estimates of numbers, variance and group sizes needed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To analyse the brain areas that are activated in our tasks, we will use a state of the art approach that requires a surgical intervention (a double virus infusion approach) that can replace the need to breed transgenic rats to control specific brain pathways, thereby minimising the number of animals required for this work.

Where possible we compare within subject data (for example in memory studies) to minimise influence of interindividual variability. Within subject experimental design is assessed using factorial ANOVAs (with the correction if the Mauchly's test of sphericity is violated). For the case of data linked to whether rats produce ultrasonic vocalisations, or not, some animals do not make the calls (for reasons still to be fully understood and which will be explored in this research project), so the data is not normally distributed and requires non-parametric or categorical statistical analysis. Behavioural data will be scored by trained observes that are rendered blind to experimental group and sex. Randomisation will be used to assign rats to the experimental groups. We have extensive experience with the data and statistical data analyses that will be used and will report the findings in line with the ARRIVE guidelines.

We have used the N3CRs Experimental Design Assistant in the past for experiments with a simple design and will refer to this to optimise our experimental design going forward, for example in planning comparison of dominance testing protocol between males and female rats.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have decided to choose a dual-viruses manipulation approach instead of breeding rats for our circuit manipulation studies. For drug-dose related studies we will pilot doses by reference to the previous literature where necessary to isolate effects to the specific behavioural readout of interest (for example ultrasonic vocalisation). In addition, we will investigate whether meaningful heterogeneity (such as dominance hierarchies) can inform the understanding of any contexts where biologically driven behavioural responses differ between animals. We will take advantage of the internal Tissue Sharing Network at the institute so that the maximum use can be gained from surplus tissue at the end of the study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The rat is a valid model species to study emotional memory and associated behaviours. Despite obvious behavioural differences, there is much evidence of conservation of the brain regions involved in emotional learning and responding between Humans and the rat. The use of the rat model allows for localised intervention studies, combined with co-relational studies of interconnectivity of small sub- regions, with a resolution greater than that which can presently be achieved in human brain imaging studies. Our previous work focused on male rats, primarily as the scientific literature has documented differences in females behaviour in aversive learning and memory given changes in oestrus cycle and differences in the sort of behaviour they show (eg. Flight behaviour in females rather than freezing, less vocalisations in females than males). However as more Human females are diagnosed with mood disorders, there is a need to better understand potential biologically driven sex differences in behaviour. Therefore, we intend to begin some select experiments in this programme of work to assess to what extent female and male rats differ in our primary tasks of interest, starting with how our method of measuring dominance in males compares to females, and then testing whether their responses to the vigilance test task are comparable to males.

We have chosen to use the method of inescapable electric foot-shock as an aversive stimulus, which is a widely-used method to model associative pavlovian learning and memory potentially at play in post- traumatic stress disorder (PTSD). We test for the associative learning by presenting the associated cue (typically spatial context, visual, auditory or olfactory cues) in a subsequent session under conditions of safety. The responses to cue that are measured are experimentally robust and consistent across experiments (cue triggered short-lived immobility called freezing, and/or ultrasonic vocalisation alarm calls). We also study the vicarious nature of learned responses to threats, i.e. how one animal learns from observing another. For these experiments an observer rat is placed with a previously trained demonstrator rat in presence of a potential threat cue. The observer learns to react from the social experience. We routinely follow conditioning with extinction protocols (presentation of cues previously associated with shock, but without shock in a context of safety) in order to study how the acquired defensive responses to the cue can be rapidly reduced, within a single session, and this protocol is analogous to exposure therapy in human patients and indeed recruits similar neural mechanisms.

We believe electric footshock is the most refined method for studying acquired aversive learning and memory because:

Firstly, electric foot-shock conditioning is a well-studied procedure where the parameters of shock intensity and duration can be tightly controlled to the lowest levels needed and are known to recruit comparable neural mechanisms in the rodent as in the Human

subject. This so called ‘threat’ conditioning learning is rapid, and the defensive responses are specific to the conditions of the experience (cues and/or context). The response of the animal can be parametrically related to the number of pairings of a cue with the foot-shock, so offers precise experimental control of the extent of any aversive experience. We and collaborators have used a limited number of pairings of footshock with a cue in a single session to produce robust and replicable responding but below engaging any panic-like escape responses. The acute response to these levels of foot-shocks are short lived, on a matter of seconds. In over 10 years of experience, we have never seen any indications of lasting distress nor physical harm after conditioning. Very often (>75% of experiments) we do follow up testing of the behavioural response to the paired cue under conditions of safety, during this session the responses start to be extinguished.

Secondly, electric foot-shock can be used as an aversive stimulus in freely moving animals without any restriction on their mobility. Other stimuli used for conditioning, such as air puff or very loud noises, recruit specific neuroanatomical circuits that differ from those recruited by a footshock.

Thirdly, the appropriate response to a threat cue in rodents is thought to be determined by perceived cue proximity and context. Therefore, we use inescapable conditioning boxes to study specifically freezing and vocalisation responses rather than escapable threat where rodents switch to an active escape and/or avoidance strategy of responses (rather than passive freezing response- thought to be used for impeding detection by a predator). Avoidance can be modelled instead without the use of foot- shocks by using an open field arena or elevated mazes.

Thus, for understanding the psychological, neurobiological and neurochemical basis of pavlovian aversive memories relevant to anxiety disorders, and particular to hypervigilance, we consider the use of mild inescapable electric shocks to represent the most refined procedure that will allow us to address our scientific questions.

In order to study innate (untrained) responses to potential threat, we have chosen to use derivatives of predator odour. The artificial derivative is a refined way to study innate responses to an ambiguous threat (distant / source not obvious) as the responses evoked by this innocuous, but aversive, odour mirror those from a laboratory conditioning task using foot-shock in the short term (freezing and USVs). However, the evidence suggests this is despite recruiting distinct neural mechanisms. We will explore why these responses persist longer (i.e. are resistant to behaviour extinction) than those evoked by footshocks (pavlovian conditioned responses). During a period of several minutes of exposure to the predator odour, unconditioned defence-related behaviour is measured. At the conclusion of testing, the animal is returned to its home-cage and can be tested the next day for retention of any associative (conditioned) contextual learning, under conditions of safety. The duration of exposure needed for experimental analysis is thus relatively short sessions (on the odour of minutes to maximum an hour). The starting concentration and duration of exposure to the predator odour will be based of the existing literature and we will conduct

pilot studies to refine the parameters to a lower limit of exposure that still produces robust levels of freezing behaviour, as defined by being significantly higher levels than evoked by baseline context or stimulus novelty exposure.

Various mazes, mostly the elevated plus maze and open field, are routinely used to measure anxiety- like symptoms in rodents. We will give brief (5-10 minutes) exposure of the rats to these mazes to measure how much they avoid the open and brightly light environment (comparable to an agoraphobia) to study how learned experience impacts these untrained (innate) responses. We performed a series of studies which demonstrated that under our conditions there was no sensitisation to repeated exposure in the elevate plus maze. Such tasks are used in a screening manner to see individual (trait) differences in conflict between the motivation to explore the environment and remaining in the perceived safety of an enclosed space. Therefore, we expect no cumulative effect on the rats' behaviour. Nonetheless, we will monitor the data and envision that the vast majority of the rats will have typically maximum 3 or 4 short trails in the mazes across the duration of the experiments.

To study the consequence of individual differences in social behaviours in rats, ultrasonic vocalisation calls will be played back to the rats for brief durations (order of minutes). This evokes short-lived changes in heart rate and behaviour (approach or avoidance depending on the nature of the vocalisation). We will also study the influence of social hierarchies on behaviour using tasks that can elucidate the hierarchy based on appetitive resource competition. The task uses highly palatable food rewards for limited access periods where they compete for access to the resource. We have previously refined this procedure to be able to carry it out without food restriction, by examining which rewards evoke the strongest motivation to consume (strawberry milkshake versus sucrose solutions) and parametrically measuring the habituation period needed to overcome any neophobia of the resource in the rats. As such this intervention allows us to reliability establish the dominance hierarchy (based on drinking time) without food restriction or stimulation of aggressive behaviours.

To test a specific requirement of discrete neurochemical systems in a particular brain region of interest, the animals may be implanted with chronic, indwelling guide cannulae targeting specific brain regions following intracranial stereotaxic surgery (localised small injection sites). The typical targeted brain areas, which compose the major classical limbic circuitry, include chiefly the amygdala nuclei, bed nucleus of the stria terminalis, hippocampus, and the prefrontal cortical regions (prelimbic and infralimbic cortex) which are arguably particularly more tractable in the rat rather than mice. The guide cannulae allow the subsequent administration of substances that modulate brain function directly to these brain regions via micro injectors. Where the effects of drug dose, or the effects of different drugs, on a specific, individually variable behaviour are being investigated, animals may receive treatment with multiple doses of drug or different drugs, with an appropriate recovery period between administrations. The 'washout' period and doses will be based on that described in the literature for the specific drug. To test functional

recruitment of a specific brain area, specialised virus-mediated targeting will be employed. Rats will undergo the stereotaxic surgery and viral vectors will be infused into the brain via stainless steel injectors to transfect regions of interest. Typically rats recover from these surgeries fully within a week. These methods offer the advantage over older approaches that permanently inactivated brain circuits (by lesion) as we can look at recovery of responses in absence of the manipulation and gain more data from each step of the protocol.

In order to test for predictive validity of our tasks we will test drugs that have the desired therapeutic effects in Humans to see whether they alter behaviour in the tasks we refine. Within such a back translation experiment, this will involve assessment of the effects of administering (acutely or chronically) substances hypothesised to modulate neuronal or glial function on a specific behavioural task. The dose and route of administration will be based on careful review of the existing literature. We have adapted our systemic injection technique to reduce restraint based on recent publications in the field. For all experiments, the rodents will be ‘habituated’ to the process and route of administration in a session where they receive a vehicle injection that allows for a within-subjects control for any effects of the procedure.

Why can’t you use animals that are less sentient?

The objectives of the research programme require measurement of behaviour in adult social animals. Although it may be technically possible to use mice to understand some mechanisms of aversive conditioning, their relative poorer social behaviour repertoire renders them a less tractable model.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The vast majority of protocols have been used in the lab before without evoking any welfare issues; however, rats will be closely monitored throughout the experiments. The mild foot shocks have been titrated down to the lowest level possible to give robust behaviour and do not cause any lasting physical effects. We have also removed the need for food restriction in our appetitive tasks by comparing and identifying the most palatable food rewards. Any surgical procedures will be carried out under general anesthesia using peri-operative analgesia and aseptic technique, followed by post- operative analgesia and regular health monitoring. If a rat shows signs of ill-health, such as piloerection, hunch posture, inactivity or inappetence it will be humanely killed, unless in the opinion of the NVS, such clinical signs can be remedied promptly and successfully using no more than minor interventions. We will continually review and refine our endpoints in consultation with our NVS/NACWO

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will continue to make use of resources such as the experimental design assistant tool, LASA guidelines (particularly for behavioural neuroscience) and the ARRIVE guidelines for publishing our work. We also make use of G*Power open access software to perform our power analyses to predict our sample sizes and systematic literature review and metanalysis tools such as the PRISMA guideline to bolster the predictions from the background of experimental design.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

My institute has developed online secure resources for updating on regular user meetings and procedure refinements, and the resources of the tissue sharing network. We will attend regular meetings to share best practice and review our standard operating procedures given any advances that could improve the animal welfare and scientific endeavours.

98. Breeding and Maintenance of Genetically Altered Zebrafish

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Zebrafish, Breeding, Genetic Alteration

Animal types	Life stages
Zebra fish (Danio rerio)	adult, neonate, juvenile, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The purpose of this licence is to generate genetically altered zebrafish (and larvae) so as to maintain breeding lines and provide animals for scientific purposes, either as larvae or to perform post-mortem tissue analysis directly from this licence, or for transfer onto other licences with appropriate permissions in place.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Genetically altered zebrafish can be used to increase our understanding of the importance of specific genes in a wide range of human diseases. By altering individual genes we can understand their role in development processes in particular their importance in models of

human cancers. An early zebrafish larvae is not a protected animal until it is capable of free feeding - typically 5 days post fertilisation.

Understanding of gene function can be done in the majority of cases using larvae before they become a protected animal.

This service licence will bring the supply to all the individual fish research groups together under one central umbrella. This, in turn, will create centralised expertise and allow for optimisation of the 3Rs.

What outputs do you think you will see at the end of this project?

The main outputs of the project will be:

- Peer-reviewed publications: in these we will share our findings with the academic community;
- Datasets: the data underpinning the publications will be shared freely online;
- Transgenic zebrafish: these are powerful tools to address a host of questions in the biomedical research field. They will be shared with any interested party so long as the appropriate permissions and paperwork are in place

Who or what will benefit from these outputs, and how?

Data obtained from this licence will be shared within the scientific community by publishing results in scientific journals following ARRIVE guidelines, presentation at scientific meetings and sharing of novel genetically altered zebrafish strains to avoid duplication.

The fish themselves will benefit from a centralised PPL as it will ensure high standards of welfare are maintained throughout their life experience and that any new improvements or refinements, whilst enabling improvement to care can be implemented more readily

How will you look to maximise the outputs of this work?

Results will be shared freely within the scientific community, novel genetically altered fish strains will be shared to avoid duplication and published in databases.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 19,500 Zebra fish

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project licence is intended to act as a service licence, in which users are able to create and maintain genetically altered zebrafish lines. To do this, it is imperative that all life stages are included.

Users will also be able to obtain life stages that are not covered by the Animal (Scientific Procedures) Act (pre-protected stages are zebrafish at less than 5 days post fertilisation where they are not capable of independent feeding) for their scientific studies. This reduces the need to use later stages that are more sensitive to experiencing a level of pain, suffering, distress or lasting harm.

It also affords them the opportunity to cryopreserve their lines, in which case adult zebrafish would be needed.

Typically, what will be done to an animal used in your project?

Animals will undergo natural mating which does not cause any adverse effects. In a small number of fish a small biopsy of the caudal fin may be taken under anaesthesia to provide cells for genetic analysis. Where possible this may be done using a mucosal swab rather than a fin biopsy. Fish may be maintained for up to 18 months of age, during which time they may be mated repeatedly for production of embryos.

What are the expected impacts and/or adverse effects for the animals during your project?

There are no anticipated adverse effects that are more than mild and transient. Pain from fin biposy will be controlled by use of anaesthesia and analgesia.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of fish will only experience sub threshold severity, a small number - less than 10% will experience mild severity associated with the anaesthetic event required for caudal fin biopsy.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals are required under this project so as to provide zebrafish for scientific purposes. Zebrafish are required scientifically to investigate interactions between individual cells and populations of cells, within a whole body setting. Where possible, zebrafish larvae will be used as a non-protected model in which to achieve the scientific purposes.

Which non-animal alternatives did you consider for use in this project?

Prior to the use of animals generated under this licence, the scientific aims will first be investigated using in vitro and in silico methods as far as possible.

Why were they not suitable?

Whilst useful in establishing simple interactions in vitro, and predicting interactions in silico, the models available are not yet suitably advanced to allow the full interactions to be modelled and predicted with certainty outside of an in vivo setting.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals estimated is based upon discussions with our researchers as to the numbers of animals they predict to need. From this we have determined the number of parents required to generate the numbers needed for scientific purposes, and maintenance of the breeding colonies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Given that the outputs from this service licence will not be numerical data, experimental design calculations have not been possible. However, the design of our breeding strategies will be dictated by user demand and will be rigorously reviewed during the lifetime of this licence.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All breeding under this licence will be stringently managed by highly experienced technicians. Careful management of the fish lines will help to reduce the number of animals produced; i.e. animals will only be produced where there is a justifiable need. Where possible, all animals bred will be shared between research groups, unless genetic status dictates otherwise.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically altered zebrafish will either be created or maintained under this licence. Only genetic alterations that at most are predicted to cause mild impact on animal welfare will be bred under this licence. Where a new genetic alteration is made, the resulting progeny will be monitored carefully during gestation and the non-protected phases to identify any mutations that could potentially impact on animal welfare. These animals will be additionally carefully assessed for any welfare impacts once they reach free-feeding stages. Any animals found to be suffering more than mildly will be killed humanely and the breeding of those animals re-assessed.

Why can’t you use animals that are less sentient?

Of the animals being generated for scientific purposes, they will principally be immature zebrafish larvae. Where this is not appropriate scientifically, then protected animals will be used, however this will be for post-mortem tissue analysis without undergoing any additional procedures. Where protected animals are being used for maintaining transgenic lines, unfortunately less sentient animals cannot be used due to the nature of the process.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Where animals will be subject to painful procedures, for example fin biopsy to obtain genetic material for genotyping, we will use post-operative analgesia to minimise any pain and suffering. We are also exploring alternative methods such as the use of swabbing the mucosal surface to obtain cells that allows for the genotyping of fish.

We have an ongoing programme looking at different tank enrichment items to benefit fish welfare.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will consult with leaders in the field of zebrafish breeding including the NC3Rs (https://www.nc3rs.org.uk/3rs-resources/zebrafish-welfare) to ensure that procedures are conducted in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We subscribe to the NC3Rs newsletter, attend 3Rs-focussed events, and interact with key individuals in the zebrafish field to ensure we stay informed of 3Rs advances. The advantage of having a centralised zebrafish breeding licence is that any advances are easily implemented leading to immediate and widespread impact.

99. Refining Methods of Resuscitation from Cardiac Arrest

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Cardiac Arrest, Defibrillation, Ventricular Fibrillation, Resuscitation, Cardiopulmonary Resuscitation

Animal types	Life stages
Pigs	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To research technologies that can be used in a simple manner to help untrained rescuers save victims of cardiac arrest. These include new methods to defibrillate and provide coaching on cardiopulmonary resuscitation (CPR).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Survival from out of hospital cardiac arrest is low (typically <10%). New technologies that can help untrained rescuers or bystanders rescue a victim of cardiac arrest have the potential to improve survival rates. This project aims to develop several new aspects of automated external defibrillators (AEDs) including CPR feedback and the methods for delivering shocks all of which can contribute to advancements in and/or proliferation of AEDs.

What outputs do you think you will see at the end of this project?

Outputs from this project will include conference abstracts, publications, new product development; advanced AED solutions, intellectual property and data to support regulatory approval of AEDs.

Who or what will benefit from these outputs, and how?

In terms of publications and academic presentations, the benefits will be realised within 6 months as per our publication targets. In addition other benefits will be realised in the medium term (during the course of this project) including marketing of new products and regulatory approvals. These new technologies will impact outcomes for patients over the longer term by improving lay rescuer CPR performance and potentially reducing the energy required for effective defibrillation.

How will you look to maximise the outputs of this work?

We will seek to disseminate knowledge through internal seminars including stakeholder workshops, national and international conferences and publication in peer review journals. Data generated from this project will be cited against any marketing claims associated with products developed as part of this work.

Species and numbers of animals expected to be used

- Pigs: 250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

A porcine model produces a good replication of the human cardiovascular system. Pigs feature a barrel shaped thorax compared to a human; however porcine models are preferred for resuscitation studies due to overall anatomical similarities. 10-24 kg pigs are a widely accepted model for paediatric cardiac arrest while 25+ kg pigs are accepted as a model for adult cardiac arrest.

Typically, what will be done to an animal used in your project?

Animals will be brought into the facility with an appropriate period of time to acclimatise prior to the start of the study. They will be housed socially in an environmentally enriched pen with the last animal in any group being held in isolation for no longer than 4 hours. Animal will have access to food until 2 hours prior to premedication, and water will be provided ad lib until premedication is administered.

All procedures will be conducted under general anaesthesia following which the animal will be euthanised. Animals will be sedated prior to induction of anaesthesia administered by intramuscular injection as a sedative. Following this the animal will be intubated and physiological monitoring including cardiovascular and respiratory measurements, will be recorded via the vital signs monitoring. A surgical approach will be made to a jugular vein and at least one artery. An introducer sheath will be placed into a jugular vein and a pacing wire will be placed into the right ventricle to facilitate VF induction a blood pressure transducer will be attached to the sheath on the arterial line. Ventricular Fibrillation may be induced via electrical stimulation and resuscitation procedures including CPR and defibrillation may follow.

What are the expected impacts and/or adverse effects for the animals during your project?

Pigs will be loaded (typically in pairs or groups) onto an approved animal trailer and transported by road (approximately 10 - 30 minutes) to the facility. Pre-anaesthetic medication (to which hyaluronidase may have been added) will then be given by IM injection. A remote (low-stress) technique will be used. The IM injection may cause mild and brief (< 5 s) discomfort. Pre-anaesthetic medication will typically produce sufficient central nervous depression, anxiolysis and muscle relaxation for the animal to be lifted onto, and moved by trolley - without resistance - to the anaesthesia induction area. Here anaesthesia may be deepened (initially) to allow the painless insertion of an IV cannula, after which anaesthesia will be induced, usually by a combined IV and, or inhalation technique. Thereafter, continuous and close monitoring of autonomic nervous and nociceptive reflexes, combined with alerting signals generated from quantitative EEG-based monitoring technologies will ensure the attending anaesthetist is always aware of

the animal's anaesthetic depth, whilst the immediate availability of additional doses of rapidly-acting anaesthetics pre-located in a venous cannula will ensure the rapid restoration of oblivion if required. The study will end once all objectives have been achieved as far as is possible within the permitted time limit. The animal will then be terminated whilst anaesthetized at the end of the study and not released for post mortem examination, or disposal, until physical examination confirms the animal is dead.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Non-recovery

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Resuscitation from cardiac arrest involves complex physiological processes that include mechanical and electrical activity of the heart. At present it is not possible to model these processes or recreate them in bench or lab studies. Pigs are the gold standard for studying resuscitation in the field due to anatomical similarities to humans.

Which non-animal alternatives did you consider for use in this project?

3D cell culture models of heart tissue are emerging and represent a promising future direction for this work, however at present they are not a suitable alternative.

Why were they not suitable?

These models do not yet represent the metabolic, structural, or electro-mechanical properties of the heart that are required to investigate cardiac arrest.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Comparison of this body of work's scope and aims with our historical work. The number of animals in our previous studies are a strong indicator of the number of animals we will use in future work.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The level of data required is ascertained in the design phase for each study. Utilising pilot studies, previous work and the vast experience across the broader team the number of animals required to obtain the required data is calculated. The key step is excellent communication across the experienced project team where the details and goals of each study are openly discussed with particular emphasis on reduction.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In our experience relatively small groups of animals, some 6-8 animals per group, are needed for a preliminary evaluation of an idea. For statistical power, particularly if establishing that there is no benefit from an intervention, larger groups of some 10–15 animals are needed. A calculation is made before every study to ensure the right number of animals is used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Porcine model of sudden cardiac arrest. All studies are conducted under general anaesthesia, so the animals are not aware of any of the procedures and feel no pain. When administering anaesthetics to the animal its windpipe could become blocked as it closes over (spasms). However, a breathing tube can be inserted straight into the windpipe to ensure the animal can continue to breathe normally while under anaesthetic. Pain during surgery will be controlled by ensuring adequate anaesthetic and pain relief is provided for each animal. The animal’s blood pressure and oxygen levels, as well as heart

rate will be monitored during the procedure to help ensure the animal remains under anaesthetic. Induction and maintenance of anaesthesia will be conducted by an experienced veterinary team

Why can’t you use animals that are less sentient?

There is no less sentient species that provides the anatomical similarity to the human cardiovascular system. Animals will be terminally anaesthetised for these studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Each animal will be closely monitored, throughout the duration of the study, for physiological status and depth of anaesthesia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will follow the NC3R guideline and conduct studies according to GLP, studies will be reported according to ARRIVE.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will read the excellent updates sent by the institution on this topic as well as partake in as many webinars and training courses are reasonably practical, I will also inform myself by reading relevant literature and will contact others including AWERB committee members prior to study start and ask for further advise. Furthermore I will maintain regular communication with NAWCO and trained husbandry/technical staff to ensure optimized animal welfare during the housing prior to procedures and during procedures is ensured.

100. Investigating the Mechanisms Obesity and its Comorbidities and its Interplay with Adrenal Dysfunction

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Adrenal, Hormones, Obesity, Metabolism, Co-morbidities

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this programme of work is to understand mechanisms that lead to obesity and its co- morbidities, with special emphasis in the development of adrenal dysfunction. For example, we will study the effect of hormones on obesity development such as adrenal hormone cortisol. Disruption of molecules or pathways that control adrenal function and metabolism gives rise to changes in body weight, body composition,

obesity, diabetes, cardiovascular alterations, cognition and related metabolic conditions which will be studied in this project.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Over the course of this project, we will investigate factors and pathways that control metabolism, especially those that affect adrenal function and obesity.

Obesity incidence continues to increase in high- as well as low-income countries, causing 2.8 million deaths annually worldwide (WHO, 2021). Moreover, obesity and its many comorbidities (diabetes, cancer , hypertension, cardiovascular disease, mental health, etc.) incurs in vast expenses of public money to tackle the management of preventable obesity-related illness. There is no pharmacological treatment available to treat obesity, in part due the lack of complete understanding and complexity of its causes. Finally, the link of obesity to other disorders such as cognition, cardiovascular imbalance and adrenal disease remains poorly understood. Importantly, adrenal imbalance and its link to obesity and associated diseases (such as cognition impairment) has not been fully explored thus far.

Taking into account all the above, undertaking the work proposed for this licence will fill substantial gaps in the knowledge regarding obesity and related diseases, with a focus on adrenal function. Our work will define mechanistic knowledge about the fundamental physiological processes which control the vital processes of food intake and energy expenditure and adrenal function. This knowledge will allow us and others to identify targets for future drug therapies, and potentially reduce the morbidity and mortality associated to obesity.

What outputs do you think you will see at the end of this project?

Over the course of this project, we will investigate mechanisms that contribute to obesity and its co- morbidities, including factors and pathways that control adrenal function. Understanding the mechanisms and being able to identify potential therapies to obesity and adrenal dysfunction will be of great impact to the individual and society as a whole. Outputs will include publications in high impact journals, dissemination and sharing of new information through meetings, congresses and conferences to facilitate data sharing and leading to collaborations for mutual benefit. Moreover, we will reach to the general public with the help of social media and the press, when appropriate, facilitating the access to the new knowledge using lay language. The project will also facilitate sharing specific techniques, skills and equipment thus facilitating further collaborations. All activities will further augment future research.

Who or what will benefit from these outputs, and how?

Increased understanding of obesity and mechanisms that lead to obesity (short-term):

In the short term, this will define new mechanistic knowledge about the fundamental physiological processes which control the vital processes of food intake and energy expenditure, both central and peripheral. In understanding such processes, we will be able to identify molecules as biomarkers or targets for future drug therapies that will combat weight gain which maybe through action on adrenal genes and molecules. In the long term, we hope to be able to design and implement strategies to prevent or reverse the development of common, serious obesity- and/or adrenal-related metabolic diseases which continue to cause substantial morbidity and mortality.

Increased understanding of adrenal dysfunction and disturbed metabolism (short-term):

In the short term, we will identify new mechanistic knowledge on the genes/molecules involved in adrenal function. This will also allow us to determine the link between adrenal function and metabolism. In the medium term, we aim to rescue adrenal function disruption and assess the effects on metabolism. In the long term, we hope to be able to design and implement strategies to prevent or reverse the development of metabolic disease.

Generate new therapies to treat obesity, its co-morbidities and adrenal dysfunction (long-term):

We aim to discover new therapies to treat adrenal dysfunction as well as obesity. Output of the research programme will be shared with the international scientific community and other interested parties such as those in the pharmaceutical industry. We will also disseminate data to the general public, patient groups, practitioners and clinicians where appropriate.

How will you look to maximise the outputs of this work?

We already collaborate widely and will continue to do so. New knowledge will be disseminated to academic beneficiaries via publication in high impact peer reviewed open access journals and presentation at national and international scientific meetings. Such activities will foster mutually beneficial knowledge exchange and collaboration between research groups which will increase impact. Moreover, we will reach to the general public with the help of social media and the press, when appropriate, facilitating the access to the new knowledge using lay language.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are a good model for human metabolism, in particular, for the study of obesity and adrenal function. Many discoveries of hormones that cause obesity come from the study of mice. This project will use mice to model human metabolism and to find new pathways and genes that have an effect on weight gain, the development of obesity as well as its co-morbidities (diseases associated with obesity) such as cognitive impairment, cardiovascular disease, fatty liver disease and diabetes. HPA axis and the factors that affect its normal function in humans are precisely replicated in mice, making rodents a good model for its investigation. Metabolism affects mammals at all life stages and hence all life stages will be studied and both sexes. This is because metabolism in females and males differ.

Typically, what will be done to an animal used in your project?

In a typical experiment, genetically modified animals will undergo assessment of their metabolism and hormone status, including those markers relevant for the assessment of adrenal function. Mini-pumps will be implanted to a small number of transgenic or wild-type animals delivering substances that will allow to study specific pathways relevant for the understanding of obesity and/or the adrenal function. We will then monitor weight, length, fat/lean mass, food intake and energy expenditure, whilst on normal diet or altered diets. For the measurement of fat/lean mass and food intake and energy expenditure, the animals will undergo a period of acclimatisation before the procedure is performed to reduce stress. Biological samples and repeat phenotyping are collected before starting any treatments and at intervals after any treatment. In some animals, we will study complications of obesity and adrenal signalling impairment such as changes in behaviour, response to stress and cardiovascular complications.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of our protocols and procedures are not expected to cause long-lasting adverse effects. However, animals can experience discomfort and pain from the injections, mini-pump insertions and/or sample collections; animals will be monitored and given pain-relief as needed. Treatments and gene alterations can change body weight and behaviour and general overall condition, which will be monitored closely. Animals showing adverse events that do not improve will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The degree of severity is expected to be moderate or less depending on the procedures we propose to use. All of our protocols will be performed on mice. Mice will be closely monitored for development of any adverse clinical signs and clear endpoints are defined in the protocols in order to minimise conditions of distress. At the end of each experiment and at well specified humane endpoints, mice will be humanely killed and blood and tissues will be collected for analyses.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our research uncovers the mechanisms of obesity and its co-morbidities in particular the affect of adrenal function on metabolism. The two are integrally linked, for example, the hypothalamo-pituitary adrenal (HPA) axis and stress hormone cortisol and adrenal androgens all have a profound effect on food intake and body weight. Due to the genetic and physiological similarities to humans and their accessible genetic manipulation, mouse models remain an important tool for the discovery of new mechanisms as well as optimisation of new therapies prior to human studies. Other species such as invertebrates, amphibians and fish do not have adrenal glands or adipose tissue depots and therefore do not share the HPA axis structure and function and fat storage physiology with humans, making them unsuitable as models for out studies. Our models involve characterisation of whole body phenotypes and their application to adrenal function and obesity, we need to study the effects in the whole animal. Hence analysis of isolated cells is useful but not sufficient to understand the function of these genes in the whole organism. In this proposal the rodent model will be used to interrogate the effects of genes in adrenal dysfunction and obesity that would not be possible in human subjects. The dissection of mechanism requires an intact HPA axis as well as adrenal and fat tissues for examination such that at present it is not feasible to completely replace the use of rodent models for this project.

Which non-animal alternatives did you consider for use in this project?

We continue to work on human and murine cell lines (adrenal and neuronal) where appropriate to determine efficacy in-vitro importance of the gene in question, and efficiency of key reagents, such as viral vectors and antibodies. In addition, we have started working

with organ-on-a-chip models to reduce the need for in vivo animal work when studying specific pathways. To identify genes and pathways that are involved in the control of adrenal function and metabolism, we will use retrospective clinical data, clinical profiling, genome wide association study data as well as data from other genetically modified mouse models that have been published. We also regularly review the most up to date published data to inform and refine the need for animal experiments including genetic data on humans. Hence, where possible we replace animals with cell-lines in key preliminary steps.

Why were they not suitable?

To dissect the control of adrenal function and metabolism, we need to characterise the effects in the whole animal. Hence analysis of isolated cells or organ-on-a-chip models are useful but not sufficient to understand the function of these genes in the whole organism. Moreover, cognition and other aspects of stress induced responses are only conveyable in living animals. In this proposal the mouse model will be used to interrogate the effects of genes in adrenal dysfunction and obesity that would not possible in human subjects. The dissection of mechanism requires an intact HPA axis as well as adrenal and fat tissues for examination would mean that at present it is not feasible to completely replace the use of rodent models for this project. We considered other less sentient species such as invertebrates, amphibians or fish but unfortunately the structure and function of their 'adrenals' is unlike that of mammals. Finally, the review of published data, although extremely useful to guide the following steps of our research, does not allow the discovery of new essential knowledge.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In our experimental design, we carry out robust statistical analysis of our pilot data and review the relevant published data to ensure that our results have the best balance between sample power and the highest quality results using the minimum possible number of animals. We aim to generate litters with both controls and experimental genotypes represented, allowing us to control for inter-litter variation and to use pairwise/repeated measures statistics, thus increasing the power of the analysis. All experimental animals will receive the same treatment from the investigator (e.g. we will perform sham surgeries, administer saline in place for drug treatment, etc) and will be housed at similar stocking density. Researchers will be blinded by genotype while performing tissue collection and cell counting analysis. Most analyses will be performed at least in 2 independent cohorts of

animals to generate overlapping data thus providing replication. In addition, we will usually perform analysis on both sexes and we expect to see replication between the sexes for most experimental outcomes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The quantity of animals used and the total number of experiments carried out will be kept to a minimum. To prevent unnecessary breeding, we keep stocks of frozen mouse sperm and embryos. In planning our experiments, we perform statistical analysis of the minimum number of mice required to observe a clear outcome. Where relevant and possible, our experiments will inform and be supported by computational tissue modelling or published pilot data that informs key aspects of adrenal function and metabolism to generate and test the hypothesis. We will use state-of-the-art non-invasive technology to determine adiposity and metabolism in an animal (metabolic chamber-calorimetry, Magnetic Resonance Imaging), which will reduce the number of animals used in this licence because they allow the follow up of the same animals in time, hence reducing the need for additional controls. We will refer to developed Experimental Design Assistant tool (EDA https://eda.nc3rs.org.uk/) and InVivoStat (http://invivostat.co.uk/) where relevant to assist in design and analysis of data from animal experiments. Finally, we will perform our experiments in animals of both sexes, reducing the need for future replication if only one sex was explored.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To ensure this we will take four approaches: 1) We rigorously monitor the breeding regiment of our stocks generating animals from the fewest breeding pairs/trios and increasing these only as experiments require. 2) The team shares animal material between several projects and communicates our animal needs during group meetings to increase the efficiency of work and minimise wastage. This also includes keeping track of stored samples and distributing them, rather than generating new samples from additional animals. 3) We generated and freeze primary cell lines from animals and use these for mechanistic and exploratory studies prior to embarking on an animal experiment 4) We collaborate extensively with colleagues within the UK and worldwide, and share animals, tissues, cells and samples with others authorised to use them. This means that others do not generate new mutants or samples, minimising animal numbers. We have benefited from others sharing samples with us.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the model organism of choice for studies in which mammalian gene function is addressed. It remains the most tractable mammalian organism for mutation and manipulation for genetic material and for subsequent phenotypic analysis. Furthermore, in bred strains used in this licence are genetically homozygous, therefore genetic background effects that complicate analysis and interpretation of outcome are minimised, and fewer animals need to be used to achieve statistical significance. The mouse genome has been extensively sequenced and characterised, and protocols for embryo and reproduction manipulations are well-established and safe for the mouse.

Why can’t you use animals that are less sentient?

Other species such as invertebrates, amphibians or fish do not have adrenal glands or the same adipose tissue depot distribution or classes (white versus brown adipose tissue) like mammals, hence cannot be used.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We predict from previous studies that over-expression/deletion of candidate genes or regulatory factors are unlikely to cause severe phenotypes. We will use established reagents and protocols where possible to minimize the unknown effects on the mice. Animals will be trained/acclimatised to those procedures where such treatment can improve the animal experience or reduce the severity of the procedure. When possible, inducible or tissue-specific constructs will be used so mice will not display a phenotype until candidate gene expression or deletion is induced. In experiments involving moderate severity protocols, we will use well established protocols and appropriate protocols for anaesthesia and perioperative analgesia will be used to avoid suffering. None of our protocols is categorised as severe.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use well established protocols and appropriate protocols for anaesthesia and perioperative analgesia will be used to avoid suffering. We will refer to the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines as well as ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines to improve the reporting of research using animals, maximising information published and minimising unnecessary studies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institute is proactive at ensuring that all involved in animal experimentation are fully up to date with the advances in the 3Rs and that these are implemented effectively during any projects. We are invited to attend annual meetings and webinars organised by the establishment and NC3Rs. We are on the NC3Rs mailing list and are updated regularly on the latest news and developments.

101. In Vivo Studies of Acid-Base Disorders

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Heart failure, Metabolic disorder, pH, Acidosis

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overarching aim of this project is to study the role of acid-base chemistry in human diseases using the most refined mouse models. This will involve establishing diseases linked to acid-base disorders, manipulating the relevant biological mechanisms using pharmacological or genetic methods, measuring the consequences of these interventions using established readouts of the affected tissues, and obtaining samples for further studies. The work will describe mechanisms and identify novel targets for diseases, ranging from relatively common heart failure and cancers to rare inherited disorders. The ultimate goal is to design new therapies that exploit acid-base balance.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Acidity is a fundamental property of intra- and extracellular fluids, commonly measured on a pH scale. Ultimately, shifts in pH arise from changes in metabolism and blood perfusion which are common manifestations of a range of human diseases. These pH changes can then feed back on biological functions, for example, by altering gene expression, protein function or even selecting certain surviving cell phenotypes. Thus, exercising control over acid-base chemistry has major therapeutic and diagnostic potential. A precedent for this line of work has been established for hypoxia, a chemical property related to pH.

Acid-base disorders have been implicated in dysfunction of various organ systems and tissues, but two prominent cases can be made for the heart, which is exquisitely sensitive to acidity, and cancer, which is driven in part by selection pressures that include acid-stress. This study plans to investigate signalling evoked by acidity, and determine how to target these processes therapeutically.

What outputs do you think you will see at the end of this project?

Socio-economic costs of the conditions researched in this project

Each year, a third of a million people in the UK are diagnosed with cancer. Colorectal and pancreatic cancers are in the top five causes of cancer-related mortality and the incidence of deaths due to pancreatic cancer among all neoplasias is growing. Treatment in most cases is surgical, which may be followed by chemo- or radiotherapy. None of the treatment options approved for clinical use exploit the acid-base chemistry of the tumour microenvironment, despite good evidence for the powerful effects of pH manipulations on cancer cell biology. It is now timely to translate the wealth of in vitro findings into tangible benefits to oncological patients.

A quarter of UK mortalities relate to cardiovascular disease. Half a million people in the UK live with heart failure, which accounts for 14% of all deaths among men and 9% of all deaths among women. Many cases of heart failure are preceded by an abnormal pattern of heart growth.

Stopping this growth process could reduce the incidence of heart disease, but many of the known targets for this are not yet druggable. Attractive novel targets for curtailing abnormal growth are those expressed at the cell surface that control ionic homeostasis, including pH.

Rare inherited metabolic diseases are often very well defined genetically, but difficult to control therapeutically. Many of these disorders have no cure. For example, propionic acidaemia (PA), a disease diagnosed in 1:100,000 births (i.e. less than 10 cases in the UK per year) is associated with childhood deaths, often due to heart problems. These

conditions involve acid-base disorders, but often through novel and poorly understood signalling mechanisms. Better characterisation of these mechanisms may deliver new treatment approaches.

Basic science discovery:

The project will test novel hypotheses related to acid-driven cancer disease progression, assess the role of pH and its regulators in cardiac dysfunction, and investigate the disease mechanisms in rare metabolic disorders. Specifically:

we will verify findings established in vitro using more integrative and disease-relevant models of cancer in mice. Fundamentally, this will address how cancers arise, what is the role of somatic evolution, and how can its trajectory be influenced with interventions.

we will link transporters related to pH control with cardiac disorders of growth and contraction. These mechanisms are difficult to study in vitro due to the nature of how the heart operates under mechanical load.

we will define how acid disorders, specifically the anions associated with acidosis, affect gene expression and protein post-translational modifications using models of acidoses. Mouse models allow tracking of responses over longer periods of time and in a more complete system without biases associated with cultured cells.

Academic and industrial dissemination:

We will publish our results in peer-reviewed journals targeting the cancer and cardiovascular fields, as well as those from the metabolic, physiological and signalling audience. We will disseminate our findings through conferences and meetings to scientists, clinicians and industrial partners. We will liaise closely with charities and patient groups.

Who or what will benefit from these outputs, and how?

The long-term major beneficiaries are patients: cancer (colorectal, pancreatic), heart failure (maladaptive hypertrophy), propionic acidaemia. Our findings in relation to disease mechanisms and the outcomes of our interventional studies may have impact on treatments.

In the medium term, our deliverables will contribute to our understanding of disease processes and benefit the scientific community as well as provide projects for training students to established researchers.

How will you look to maximise the outputs of this work?

We collaborate extensively across the research community, facilitated through consortia established locally and nationally. We engage with patient groups and hospitals providing

care. These interactions help to inform the design of experiments, and expedite efforts to translate our animal-based findings to clinical use. As per standard process, all our findings will be disseminated by presentations and publications.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In vitro studies can provide a wealth of information about disease processes, but some of the more pressing questions that directly relate to human health, require experiments on more complete systems. For conditions that affect multiple systems, a complete organism must be studied. In the case of conditions that require a particular context (e.g. tumour growth or flow of blood), or need a long period of time to develop (e.g. more chronic diseases), there is currently no suitable alternative to laboratory animals. For this project, we propose to use mice that are either wild-type or genetically modified to (i) support tumour growth (i.e. immunocompromised) or (ii) lack a particular transporter or enzyme that is related to the disease mechanism. Mice are well characterised and conducive for a range of measurement methods (e.g. imaging). The metabolic pathways, genetics and physiology of mice are sufficiently similar to humans to draw valid conclusions, particularly in light of the project's scope. Mice, unlike other species, are conducive to genetic manipulations (e.g. knockout) which means that models of human inherited diseases can be established as well as models for selective inactivation of genes under investigation. The welfare of mice has been well studied, thus experiments can be refined to cause as little harm as possible, whilst delivering high quality data. Experimental data will be collected from adult mice, typically 8-20 weeks of age, because at this age, the disease phenotype of interest becomes apparent and methods for its study are established.

Typically, what will be done to an animal used in your project?

A disease state will be induced in the animals in order to study the human condition. This will involve injecting cancer cells under the skin to grow tumours, deleting genes that play a role in diseases, or introducing chemical agents (e.g. drugs) or physical interventions (e.g. a band around the aorta) that evoke cardiovascular disease. The mice will be studied over their life time, up to adulthood. During this time, samples of blood may be collected, and tissue function growth may be assessed (e.g. dimensions of tumours or heart contractions). After humane killing, tissues may be collected for further experiments or measurements. In preparation for harvesting these biological samples, animals may be

injected with suitable substances. Mice will not be kept for longer than 20 weeks, and regular welfare monitoring will be implemented.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals may develop tumours which may metastasise to other organs. The tumour burden will be monitored so that it does not exceed the allowable limit. Changes to genes that cause human disease will also produce a similar condition in mice. Signs such as weight loss will be monitored to stop the disease process from causing excessive harm. Interventions that affect the heart may result in cardiac dysfunction, but this will be monitored before the onset of failure.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: 50% moderate, 47% subthreshold, 3% mild.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The scientific objectives of this project relate to cancer and heart disease. The molecular details of pathological processes involved in these diseases can be studied in vitro, however, a more complete and unbiased approach must consider the context (e.g. environment in which cancer cells survive and signal, the flow of blood that the heart must manage), which simply cannot be replicated reliably in a dish. Many of these pathological processes also require long periods of time to develop fully, a timeline that is often outside the scope of tissue culture. Diseases of cancer and the heart must be considered in the integrated context of the whole organism, because treatments must be effective in patients.

Thus, there is a need to perform at least a selection of experiments in laboratory animals, such as mice, that replicate the major aspects of humans and provide a means of interrogating and interfering with the disease process.

Which non-animal alternatives did you consider for use in this project?

Cancer research commonly uses cell lines in culture to study genetic mutations and their effects on simple functional readouts. Some degree of insight into cell-environment interplay can be replicated in vitro, albeit with limitations.

Disease processes in cardiac cells can be studied in isolated and cultured cells. These include disorders of growth and contraction, albeit over short periods of time, compatible with culture conditions.

For certain research aims, it is possible to use computational modelling to simulate biological processes, based on parameters that had been characterised in previous studies. In as much as possible, we will use such in silico approaches to eliminate animal experiments where they are not essential. Models are, however, only as accurate as the data they are supplied with and inevitably need to be calibrated and checked with new data, often requiring the use of animal tissues.

Why were they not suitable?

Many aspects of cancer research use cell lines in culture, which sometimes are considered as alternatives to animal studies; however, these should be considered as complementary as they address different types of scientific question and should be performed in parallel. Some progress has been made to grow organoids, but these still lack the true environment of the host's stroma and vasculature that are crucial for cancer development. Moreover, the chemical milieu of tumours is difficult to replicate accurately in vitro, and the best approach is to allow cancer cells to develop their own milieu in vivo.

Many aspects of cardiac physiology can be studied in vitro, but cardiac cells are difficult to maintain in culture for periods that reflect the timeline of pathology. Moreover, the mechanical loads imposed on cultured cells will not replicate those in vivo. The neurohormonal and physical environment of the heart is critical for disease, and these aspects cannot be readily replicated in vitro, without biasing the results or producing flawed interpretations. Thus, at least some aspect of cardiac research must involve the intact cardiovascular system of a living animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used has been estimated on (i) the basis of colony size required to yield the number of animals with a particular trait and (ii) the number of procedures required to attain significance in experiments, based on power calculations. These power calculations are based on data obtained from previous studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Many of the procedures described in this project have been refined to produce a maximal yield of data per animal. Thus, where applicable, time course or paired experiments (control and experiment group) are performed on a single animal to reduce between-individual variation. Protocols have been designed to extract multiple aspects of the disease per animal, e.g. at the end-point, blood, heart and liver would be obtained for some experiments. The techniques used, such as aortic banding, have been chosen strategically to produce consistent outcomes, which improves resolving power and keeps animal numbers low. Genetic drift will be monitored by genotyping animals after several generations; if necessary, a colony can be re-established from stored sperm.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

A series of ‘enabling experiments’, performed in culture-dishes, will first identify the conditions and types of cells that are most appropriate for testing our hypotheses in animals. Only the experiments that are most likely to produce useful outcomes will be extended to animal studies. Colony management will use an in-house database which, among other features, will monitor animal weight. The system has been well tested by staff.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The subcutaneous growth of tumours will use mice that cannot mount an immune response and thus will not produce a painful inflammatory response. Tumour burden as well as signs of metastasis will be monitored to ensure animals do not suffer excessively. Cardiac dysfunction will be evoked by chemical or physical means that produce hypertrophy but not heart failure. Signs of heart failure will be monitored to prevent animals

from succumbing to cardiovascular failure. Studies of the rare human disease, propionic acidaemia, will use a gene construct that has low, rather than absent, catalytic activity, i.e. result in a milder form that permits survival until adulthood.

Why can’t you use animals that are less sentient?

Studies of tumour growth require a mammalian system that features a vasculature and cellular environment that is relevant to the human setting. To resolve tumour growth, it is also essential to inject an adequate cell count, which then necessitates the use of appropriately sized animals. Additionally, it is desirable that the animal can support the growth of cancer cells for up to several weeks, which is essential for slow, somatic evolution to meaningfully influence tumour cells. Together, the most refined choice for an animal is a small mammal, like a mouse. Mice have the specific advantage that immunosuppressed genotypes are available. These are valuable as they allow tumours to grow without evoking an immune reaction.

Cardiac physiology requires a mammalian system with the familiar layout (e.g. 4 chambers) and a physiology that closely resembles human hearts. The derangements on the heart may take weeks to develop, thus terminally anaesthetised animals are not suitable. To trigger heart disease, some interventions will involve the aorta, so a mammalian layout of the vasculature is essential. The most refined choice for these experiments is a small mammal, like a mouse. Mice have the specific advantage of being conducive for genetic alterations either for producing an animal model of a human disease or to inactivate key genes involved in disease processes.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Peri-operative analgesia will be administered during and after surgery. Pain will be inferred from behavioural cues. Adverse changes such as weight loss will be addressed with appropriately prepared food.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Publicly available resources are accessed on www.nc3rs.org.uk, https://norecopa.no, and https://www.lasa.co.uk. Advice will be sought locally from the named veterinary surgeon and named animal care and welfare officer.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Gold standard meetings are held 3 times a year at the establishment to ensure communication about 3R opportunities. Regional 3Rs managers and Named Information Officers will be consulted throughout the project to seek advice on better methods, models,

and procedures. Additionally, information will be sought from www.nc3rs.org.uk and https://science.rspca.org.uk

102. Development, Optimisation and Validation of Small Animal Imaging

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

In vivo Imaging, Refinement, Development and Validation

Animal types	Life stages
Mice	adult, aged
Rats	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to develop, optimise, and validate in-vivo small animal imaging techniques as biomarkers to evaluate physiology, detect pathology, and monitor the effects of therapeutics

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Medical imaging techniques such as magnetic resonance imaging (MRI), computed tomography (CT), and ultrasound (US) are fundamental to modern healthcare systems. They provide doctors with diagnostic information informing on the presence of disease, its severity, its location and involvement with surrounding tissues. These techniques can provide anatomical and/or functional information about the organ of interest. Anatomical scans are pictures of organs that display the different structures of interest, for example a tumour or stroke. Functional scans are pictures of the tissue of interest that display a functional tissue property such as blood flow to the organ.

The process of developing a new imaging technique for human benefit is a lengthy one. New techniques must be tested to ensure they produce reliable results that do not depend on the type of scanner, day, or conditions of the scan. Preclinical testing provides the ideal platform for this type of development activity, due to the availability of highly specific rodent models of disease and ability to perform tightly controlled experiments for assessment of reliability.

Further to providing new diagnostic tools for clinical medicine, medical imaging techniques are useful in rodent studies to understand disease mechanisms and/or to monitor the effects of novel therapeutics. Medical imaging techniques also beneficial for animal welfare as they provide a non- invasive way of studying the function of tissues.

This project aims to develop new techniques to measure damage to the blood vessels and brain tissue associated with dementia and cancer treatments. These conditions affect huge numbers of elderly people, creating an enormous economic burden on society. Developing new clinically translatable techniques capable of diagnosing these conditions will help improve outcomes for these patients.

What outputs do you think you will see at the end of this project?

The primary output will be improved imaging capability through establishment of multiple new imaging techniques. We will aim to share these imaging techniques with other users at our institution and further afield to avoid duplication of efforts, helping to reduce animal use.

Who or what will benefit from these outputs, and how?

The following groups will benefit from these outputs:

The animals. Development of new imaging techniques will replace more invasive techniques and reduce scan durations.

Human patients: Imaging techniques developed in this project will eventually be translated to diagnose human disease.

Researchers and clinicians studying vascular and tissue damage. The project will provide new methods for more sensitive and specific study of these properties.

How will you look to maximise the outputs of this work?

We will maximise outputs through sharing of imaging techniques with other researchers at our establishment and further afield including other universities and industry. We will disseminate our methods via regular presentations at national and international conferences.

Species and numbers of animals expected to be used

- Mice: 1150
- Rats: 1150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice and rats have a developed nervous system that has sufficient similarity to human anatomy. Adult rats and mice will be used as opposed young animals because organ structure and function is fully developed. Rats are particularly advantageous since they have larger organs and will tend to produce higher quality data than mice, thus we will use rats instead of mice when developing techniques that are limited in signal strength. Development of medical imaging techniques using lower species (e.g. zebrafish) is not possible due to the much smaller size of organs.

Genetically modified animals or animals with inherent or induced cerebrovascular disease will be used when we need to test if new imaging techniques are sensitive to specific pathology.

Typically, what will be done to an animal used in your project?

Animals may receive radiotherapy to the brain or other organs prior to imaging.

Genetically altered animals may be bred and aged. Hypertensive animals may be purchased and aged until they develop high blood pressure.

We may surgically place a clear window at the surface of the skull to enable live microscopy of the surface of the brain (<5% of procedures). We may introduce needle into

the tail vein (50-80%) or directly into the brain (<5% of procedures) in order to administer imaging contrast agents.

Imaging sessions will typically last between 30 mins and 3 hours in duration. Before or during the scan, animals will be injected with contrast agents or dyes or compounds to alter physiology. The mixture of gases used may be altered during the scan to change physiology for validatory purposes or as a contrast agent. Animals may be repeat scanned up to a maximum of 10 times with a minimum of 24h between scans. Blood sampling may be performed.

Following imaging, animals may undergo behavioural testing then will be humanely killed for ex-vivo analyses.

What are the expected impacts and/or adverse effects for the animals during your project?

Radiotherapy may lead to transient reddening of tissue.

Implantation of clear windows in the skull may result in transient localised inflammation.

No adverse effects are expected from imaging apart from those associated rarely with general anaesthesia including death due to anaesthetic intolerance (~5-10 in 1000) or repeated imaging which may result in weight loss (5-10%) due to stress and repeated prolonged anaesthesia.

Injection of contrast agents or compounds may occasionally lead to adverse reactions (possibly resulting in animal death), but this will be limited via use of published dosing, preparation, and injection protocols where available. Repeated tail vein cannulation may damage the tail, leading to necrosis.

Behavioural tests may induce mild stress for a short period.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity is moderate for all animals.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal experimentation provides the ideal platform for development of imaging techniques, due to the availability of highly specific rodent models of disease and ability to perform tightly controlled experiments for assessment of measurement reliability.

Which non-animal alternatives did you consider for use in this project?

There is a certain amount of non-animal work that can and will done (e.g. on non-animal test objects made of gelatin or from cultured cells) to carry out initial scan optimisation, and we will aim to replace the use of animals wherever possible. Test objects can be constructed that mimic flow through a blood vessel and will be used to provide early testing of flow measurements. Use of cell cultures will be used to test measurements of cell permeability where practicable. However, these test objects lack the complexity needed to fully test new techniques and eventually animals will be required.

Why were they not suitable?

These non-animal systems are useful for very early and basic testing of new methods prior to applying in live animals but fundamentally cannot provide the required level of complexity to accurately mimic the function and structure of live tissues.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of mice and rats to be used throughout the project is calculated based on considering the type of animals needed, the expected number of experiments and the expected number of animals per experiment.

Up to 300 genetically altered mice or 300 genetically altered rat will be needed to validation purposes.

We will develop approximately 10 new imaging techniques and validate 6, per year per species, across all imaging modalities.

For development experiments, we will use approximately 3-5 animals. For validation experiments, we will estimate approximately 5-10 animals per group.

Together, these considerations lead to estimates of 1150 mice and 1150 rat.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will always aim to use the minimum number of animals required to for the purpose being investigated. The specific experiments to be undertaken will be guided by the imaging technique being developed, and appropriate sample size calculations with be conducted based on prior data either from publications. When data does not exist, we will acquire pilot data to inform sample sizes. We will seek expert statistical input and utilise rigorous experimental design (e.g. NC3R's EDA) in the conduct of all experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Dynamic imaging before and after injection of a contrast agent or compound will be used where possible to remove the need for a control group. Where possible, historic data will be used to inform sample sizes. Otherwise, an initial pilot/exploratory study will be used to generate data to test feasibility of the approach and provide initial estimates of effect sizes and data variability . Important experimental results will be repeated and validated via follow-up experiment to minimise the likelihood of spurious nonreplicable results by other groups. Numbers of animals used in each study will be monitored to capture drop-outs or any requirement for a positive control group. Required numbers of animals per protocol will be updated as more recent data becomes available.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Where possible, radiotherapy using gamma-radiation given under restraint rather than anaesthesia. Image-guidance will be used to target the radiation field, avoiding unnecessary radiation dose to normal tissue effects.

For all experimentation, we will use naive mice or rat where it does not impact the scientific objective. For studies requiring use of genetically altered, experimental models of cerebrovascular disease, or irradiated animals, the models that will provide the necessary pathological changes at the youngest age and with mildest phenotype will be used. All

imaging and injections will be performed under anaesthesia. During imaging, respiration and temperature will be monitored. Injections will be given using an appropriate sized needle.

Why can't you use animals that are less sentient?

When setting up and optimising imaging techniques for the first time which may require long scan durations, we will use animals that are terminally anesthetised. Adult mice and rats will be used, including genetically modified animals when necessary. Adult rats and mice will be used as opposed young animals because organ structure and function is fully developed. Use of less sentient species (e.g. zebrafish) is not possible due to the much smaller size of organs.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

A key objective of this licence is to optimise imaging techniques. In practice, this results in shorter anaesthesia durations for the animal but will also result in other benefits such as reductions in total injected volume (for example by using the shortest injection line possible).

Imaging procedures will be regularly reviewed (monthly) and areas of potential refinement identified. Animals will be closely monitored for adverse effects and appropriate steps will be taken to minimise suffering, pain or distress. Use of tools such as the grimace scale will be used to assess the condition of the animals. Wherever necessary appropriate anaesthesia will be employed to reduce the suffering and distress caused to experimental animals. Appropriate analgesic regimes (as advised by the NVS) will always be used for pain relief and for all protocols the earliest endpoints will be used where possible.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3Rs website contains the most up to date resource and will be regularly checked for updates to best practice. When injecting contrast agents or pharmacological agents, we will consult the joint consortium guidance (see protocol steps) for the most appropriate dosing and injection routes.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will remain in constant contact with our establishments animals facility personnel including the Named Training and Competency Officer (NTCO), Named Information Officer (NIO), and Home Office Liaison Contact (HOLC) to ensure I am as up to date as possible with changes in regulations and procedures. Any opportunities for additional training or CPD will undertaken without delay. We will also regularly consult with the NC3Rs Regional

Programme Manager and check the NC3Rs website on a regular basis for updates to best practice concerning injections and refinements to procedures.

103. Mouse Models of Human Genetic Disorders of Growth and Innate Immune System Activation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

growth, brain size, DNA replication/repair, innate immune response, autoinflammation

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to investigate how mutations that cause human disease can lead to dwarfism, microcephaly (a small brain) or autoinflammation. Through this research we will learn more about how body and brain growth is regulated and how a healthy organism avoids harmful inflammation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Huge differences exist in the size of different mammals, with a 100-million fold difference between the smallest and largest. What determines this is poorly understood. Our laboratory has discovered mutations in many genes that cause undergrowth of body and/or brain in human patients. Defining how these mutations cause growth defects will help us understand how growth and the size of our brains is regulated.

One way in which the body protects itself against viral infections is through activation of an innate immune response. Although this helps to defend the body, the resulting inflammation can also be harmful. In certain genetic conditions, sterile inflammation occurs: there is no virus, but there is inflammation. Defining the mechanisms underlying these so-called autoinflammatory disorders will help us understand how the innate immune response is regulated to defend against infection, whilst avoiding aberrant activation.

What outputs do you think you will see at the end of this project?

This project will primarily generate outputs in the form of peer-reviewed publicly-accessible research publications in scientific journals.

Who or what will benefit from these outputs, and how?

The immediate beneficiaries of this project will be scientific researchers working in this field. These impacts are likely to be continuous throughout the life of the project.

Medium-to-long term impacts, some of which are beyond the timeframe of this project, include better predictions of whether some types of inherited mutations will cause disorders of growth or autoinflammation or not, which has the potential to impact on patients.

In the longer term, a better understanding of the mechanisms that regulate the innate immune response may lead to possible therapeutic approaches to benefit patients suffering from autoinflammatory disease. This might extend to benefitting wider human populations, as findings could have potential relevance to more common autoimmune diseases and/or the ability to limit the negative impact of inflammation associated with viral infections.

How will you look to maximise the outputs of this work?

Output visibility will be maximized through open-access publications and publicity through conventional and social media channels. Outputs will also be maximized through presentation of data and results at scientific meetings and conferences in this research field.

Raw and processed data will be made available in public databases to allow other researchers to study and build on this work, and to identify any links that we might not

have been able to find ourselves, potentially using approaches or techniques developed subsequently.

Genetically altered lines developed in this project will be made available to other researchers, and genetically altered lines that are likely to be in high demand will be deposited in appropriate repositories (e.g. MRC Harwell hub of the European Mouse Mutant Archive).

Dissemination of new knowledge that has potential to be translatable to human genetic disease, including novel causes (new mutations and/or new genes) of heritable disorders, will be through clinical colleagues, not least those working in clinical genetics.

Species and numbers of animals expected to be used

- Mice: 10000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mouse is a good model to study genetic causes of undergrowth and autoinflammation, because it is developmentally and physiologically similar to human, with well-conserved and well-defined pathways. Also, there is good availability of reagents such as antibodies that are used to study the cellular processes involved in mice. The ease of genetic manipulation of this species, and the relatively short generation time are also advantageous.

This work will use mice containing genetic alterations in genes that affect pathways impacting on growth and innate immune responses. These animals will allow us to understand how growth is regulated, how inappropriate immune responses are normally avoided, and how these processes can be influenced. Non-genetically altered animals born to the same parents will be used as controls.

The choice of life-stages used is mainly determined by the time points at which clear differences can be observed between genetically altered mice and non-genetically altered controls. In all cases, the earliest time point at which this becomes apparent will be chosen. In relation to growth this will

generally mean working with fully grown (adult) animals to allow maximal differences to be observed. In terms of autoinflammatory disorders, this will vary between different strains and will depend on the onset of clinical symptoms.

Typically, what will be done to an animal used in your project?

Most (>90%) of the animals used in this project will be used in breeding and maintenance procedures to generate mice with the desired genetic modification. These animals will typically be killed using a schedule 1 method, and tissues dissected post-mortem for subsequent analysis by molecular techniques.

Some mice (<10%) will experience interventions prior to killing and tissue collection e.g. injection with labelling agents to mark active DNA synthesis up to 48 hours prior to tissue collection.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority (>65%) of animals used for this project will experience breeding and maintenance protocols but will not experience adverse effects given the nature of the genetic modification.

Some animals (~20%) will experience mild adverse effects due to the nature of the genetic modification (e.g. mild growth restriction that does not impact on the ability of the animals to feed or drink, or detectably alter their movement or behaviour).

Some animals (~10%) will experience transient mild threshold pain during injection of substances (e.g. intraperitoneal injection with labelling agents), due to the injection itself.

A small number of animals (<5%) may also experience moderate adverse effects due to the nature of the genetic modification. Signs of ill health resulting from inflammatory disease may include shortness of breath, hunched posture and weight loss. The duration of this will be kept to a minimum to avoid unnecessary suffering.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority (>65%) of the mice used in this project are expected to experience sub-threshold levels of severity.

Some mice (~30%) used in this project are expected to experience mild levels of severity (mild phenotypes associated with genetic alterations or injection).

A small number of mice (<5%) used in this project are expected to experience moderate levels of severity (moderate phenotypes associated with genetic alterations)

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project uses mice as an experimental model to investigate how organism and organ growth is regulated, and how inappropriate innate immune system activation is prevented, to increase our understanding of human genetic disorders of growth and autoinflammation. Some of the biological pathways investigated in this proposal are mammal-specific, and mouse is the most experimentally- accessible mammal that can be used for this work. These animal studies will provide valuable data about the mechanisms that underlie pathophysiology, which could not be gained from studying cell lines or human patients.

Which non-animal alternatives did you consider for use in this project?

Cell-free in vitro models, cell line models, and yeast were, and will continue to be, considered during the course of this project. This project is part of a larger programme of work that will also incorporate structural protein predictions, biochemical analysis of purified proteins, and clinical data from affected patients. The animal use within this larger programme will model and validate key findings from in vitro experiments and clinical data.

In our work, we use biochemical studies with purified proteins to model the impact of disease mutations on protein structure and function. In addition, where possible, we determine the impact of equivalent mutations in budding yeast. We also make widespread use of fibroblast cell lines derived from patients, genome-edited mouse ES cells with mutations of interest, as well as human iPS cells and genome-edited human cell lines. Pluripotent cells are used to determine both the direct cellular consequences of disease mutations and how this changes in more differentiated cells, e.g. through the use of differentiation into neural stem cells, neural rosettes or brain organoids. Notably, some of the mechanisms that will be studied in this project build on information obtained from work from some of these alternative systems, but the in vivo setting achieved through the use of mouse models allows the investigation of pathophysiological consequences, in particular those impacting on organism/organ growth and innate immune system regulation.

Why were they not suitable?

In vitro biochemical models (e.g. reconstituting protein complexes) provide some insight but likely still lack key components and are restricted in scope.

Whereas yeast and cell line models can be used to study certain molecular mechanisms, they cannot model all aspects of pathophysiology associated with the observed genetic

defects (e.g. those impacting on brain growth or the immune system). Some genes we study are mammal-specific and cannot be studied in yeast at all.

Although cell line, non-animal and in vitro models in isolation are not particularly well suited to addressing some of our biological questions, the animal use in this project is part of a larger programme of work incorporating iterative cycles of testing findings from these models in vivo, and using in vivo observations to develop models for the mechanisms that are relevant to the in vivo physiology.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are based on similar projects currently undertaken on an existing project licence that is due to expire.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The majority of the animals used in this project are used in breeding and maintenance protocols to generate experimental animals of the desired sex and genotype, which will generally be examined (phenotyped) post-mortem.

I have consulted with a statistician to determine appropriate sample sizes and experimental design to associate genotype with phenotypic effects in these cohorts of experimental and control animals. Data from previous phenotypic analyses were used to generate estimates of data distributions and effect sizes to facilitate those sample size calculations.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Cell line models, structural predictions, and biochemical assays will be used where possible to design and test specific types of genetic alteration, or to test functionality of fusion proteins, to identify the key genetic alterations to introduce into mice. This will reduce the number of mice used by limiting the genetic alterations studied to those that are likely to be most informative.

The most efficient breeding strategies will be used to limit the number of animals used. Genetically altered lines will be cryopreserved to facilitate sharing and distribution of these

lines, and to limit breeding and maintenance once all experimental cohorts have been collected.

Multiple phenotypic assays can typically be performed on tissue isolated from each animal, and some material frozen for use in multiple molecular assays over long time scales, reducing the number of animals needed to generate significant mechanistic insight.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Most of the animals used in this project (>90%) will experience breeding and maintenance protocols, but will not experience any additional procedural intervention, and the majority of these will therefore experience minimal distress or harm. Some of the genetic alterations will cause mild adverse effects (e.g. mild growth restriction that does not impact on the ability of the animals to feed or drink, or detectably alter their movement or behaviour). A small number of animals (<5%) will experience moderate adverse effects as a consequence of the genetic alteration (e.g. clinical features of autoinflammation), but our aim is to rapidly define the time point of onset, to ensure that the humane end point precedes moderate adverse effects, avoiding unnecessary suffering. The genetically altered tissues that will be obtained from these animals will be obtained post-mortem after killing by a schedule 1 method.

Some animals will experience additional procedural interventions (e.g. injections of compounds to study in vivo DNA synthesis) prior to killing by a schedule 1 method and tissue collection. This is expected to cause mild and transient discomfort, but is not expected to cause lasting harm to the animals.

Why can’t you use animals that are less sentient?

The life stages used for these experiments are largely dictated by the time points at which measurable phenotypes emerge. In some cases undergrowth of brain/body can be detected at the embryonic stage. However, in most cases the differences are small and the relative difference to non-genetically modified controls is more pronounced in adults. Similarly, autoimmune/inflammatory phenotypes often only become detectable in adults.

Animals that are less sentient (e.g. nematode worms, fruit flies) have no substantial brain structure compared to humans/mice and therefore would not allow the mechanisms underlying microcephaly to be studied in sufficient detail. Similarly, most of these organisms have differently regulated immune systems and would provide limited insight into mammalian immune pathophysiology.

Most of the animals used in this project (>90%) will experience breeding and maintenance protocols, but will not experience any additional procedural intervention. The genetically altered tissue that will be obtained from these animals will be obtained post-mortem, usually after killing by a schedule 1 method.

However, occasionally, terminal anaesthesia may be used to allow large quantities of blood to be collected or tissue architecture to be maintained by perfusion fixation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The majority of the animals (>90%) will only experience breeding and maintenance protocols. Refinements associated with this protocol include environmental enrichment, and implementation of non-aversive mouse handling.

Intraperitoneal injections (e.g. to label newly synthesised DNA) will be refined by implementation of non-aversive mouse handling.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs - Responsibility in the use of animals in bioscience research https://nc3rs.org.uk/3rs-resources/responsibility-use-animals-bioscience-research Medical Research Council - Guidance on research proposals involving animal use https://www.ukri.org/councils/mrc/guidance-for-applicants/proposals-involving-animal-use/

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Annual PIL refresher courses run by the Establishment will help keep me, and other PIL holders implementing this project, informed about advances in 3Rs.

Experimental interventions (e.g. injections with labelling compounds) will be authorised locally by the Named Veterinary Surgeon at the Establishment through a time-limited Experimental Request Form (maximum duration 1 year), which will allow timely implementation of 3R advances in these regulated procedures.

104. Early Diagnosis and Treatment of Pancreatic Cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Pancreatic, Development, Therapy, Prevention

Animal types	Life stages
Mice	juvenile, adult, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to better understand the early events that promote the development of pancreatic cancer. This will help us to diagnose and treat pancreatic cancer at an earlier stage.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Pancreatic cancer carries a dismal prognosis, with less than 5% of people who are diagnosed living beyond 5 years. This has changed little since 1970. Pancreatic cancer is set to become the second leading cause of cancer death in Western countries by 2030. Our work aims to better understand how pancreatic cancer develops, and also to identify better therapies.

What outputs do you think you will see at the end of this project?

Improved understand of the key mechanisms that contribute to the development of pancreatic cancer.

Test the efficacy of new therapeutic combinations to treat pancreatic cancer.

Contribute to the evidence available to the scientific community through publication, presentation as well as sharing of findings with collaborators.

Who or what will benefit from these outputs, and how?

The wider scientific community will benefit from our improved understanding of pancreatic cancer genesis. This will be of benefit to those aiming to identify pancreatic cancer at an earlier stage. In addition, those aiming to reduce the chance of developing pancreatic cancer with preventative strategies (e.g. drugs targeting inflammation) will benefit from our output. These benefits are likely to be observed over the longer term.

The work we carry out that aims to identify new therapeutic strategies is more likely to translate into improved outcomes for patients in the medium term. This work is translational in nature and the data generated from animal experiments will inform and justify early phase human trials.

How will you look to maximise the outputs of this work?

We have forged strong collaborations with pancreatic cancer researchers from across the United Kingdom. We are able to share all of our data which strengthens each individual group's understanding and also reduces duplicity. We meet regularly in order to share data and have already demonstrated procedural refinement due to these collaborations. We will also share our data by presenting at relevant national and international meetings, publishing in peer reviewed journals, and ensuring that 'negative' data is made available either through publication or collaboration.

Species and numbers of animals expected to be used

- Mice: 7400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will be using adult mice in order to test new treatment strategies for pancreatic cancer. As mice share a large part of the genome with humans, these models provide an ideal system for studying the effect of drugs on tumours. In addition, the mice have a fully intact immune system, meaning that we can test immunotherapy based drugs. We will also be breeding mice with specific genetic alterations. These alterations mean that the mice develop pancreatic tumours. This process closely resembles the process observed in humans. This provides a high fidelity model that is more easily translatable to humans.

Typically, what will be done to an animal used in your project?

Genetically altered mice of a type that develop spontaneous pancreatic tumours may be bred. We will test the DNA of each mouse in order to determine if they have inherited the genes that cause them to develop pancreas cancer. Mice who are positive for these genes will be kept for up to 15 months until they develop tumours. When mice from these protocols develop tumours, the tissue will be used in the laboratory for further experiments.

This study involves the use of scans to determine if a mouse has developed a tumour. It can be difficult to detect small tumours inside the abdomen of mice, therefore we will use scans to check whether tumours have developed. These scans include ultrasound or CT scans. This allows us to determine how long it has taken for each mouse to develop cancer in its lifetime.

In some circumstances, we will use genetically altered mice for additional experiments. These experiments may involve an operation to implant tumour material or other substances that affect tumours growth directly into the pancreas or the spleen (this then leads to the development of liver metastases, mimicking the spread of pancreas cancer). This is likely to occur in genetically altered mice who have a predisposition to pancreatic cancer. We will then test whether exposure to additional substances increases the rate of tumour development. Finally, there is some evidence to suggest that inflammation may contribute to the development in pancreatic cancer. Human patients with pancreatitis are at increased risk of developing pancreas cancer. To mimic the inflammation we will use a chemically induced pancreatitis model. This provides a model to compare the differences in immune cell populations between tumours and 'benign' inflammation.

In some experiments we will inject tumour cells into the pancreas or under the skin. Tumours will develop and we will use scans to monitor their growth. We will use treatments, including radiotherapy, to test the effect on tumour growth.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice that are genetically altered may develop pancreatic tumours. These tumours can produce symptoms of abdominal bloating, weight loss and lethargy. When mice exhibit

these symptoms they will be humanely killed. Mice that undergo implantation of tumours (under the skin or into the pancreas) may also exhibit these symptoms. With tumours under the skin, there may be instances of skin ulceration. When this is determined to be significant or does not heal, mice will be humanely killed. In all instances tumours will be monitored using scans, and we aim to end the experiment before these symptoms develop.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice

- Sub-threshold 20%
- Mild 20%
- Moderate 60%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We have gained valuable data over 10 years of research regarding the biochemistry and cell biology using cell culture techniques, however, living organisms are complex combinations of multiple interacting tissue types, which cannot be fully recapitulated in vitro. As such it is necessary to move into an in vivo system to truly validate the function of these proteins in tumour progression. Mice share 98% of their genome with humans making it relatively straightforward to apply mouse genetic research to human disease and mouse models of pancreatic cancer to closely recapitulate the human disease. Breeding of genetically altered mice is however necessary to generate pancreas tissue carrying the required genetic alterations. This will allow us to potentially replace additional breeding by being able to combine genetic/epigenetics in vitro prior to implantation.

Which non-animal alternatives did you consider for use in this project?

Where possible we will use organoid cultures derived from the pancreas of post-mortem animals. Organoids accurately recapitulate the physiology of the pancreas and can be serially passaged and therefore used for multiple experiments replacing the need for

animals. Where possible, we also use clinical samples to test our hypotheses, but this relies on the availability of biopsies, and will run concurrently with the studies proposed here.

Why were they not suitable?

The interplay between tumour cells and the microenvironment is crucial to therapy response. Therefore, it is necessary to study the response to treatment within a physiological context due to the impact of crosstalk between the tumour and its surrounding environment. Although co-culture methods are evolving, it is currently not possible to assess the outcome of therapies on the tumour and host environment using tissue culture models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have included breeding and experimental protocols in this licence. I have experience using an existing licence over a 5 year period, as well as extensive pilot data from collaborators. Our experience of breeding protocols over the last 5 years has allowed us to estimate our projected requirements. This is also the case for protocols 2 and 3 where significantly more experience has allowed us to accurately estimate projected numbers. We have also taken the COVID pandemic into account when considering our previous and anticipated usage.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We refer to the NC3R experimental design assistant when planning in vivo experiments. In addition to this, we ensure that our work complies with the PREPARE and ARRIVE guidelines (https://norecopa.no/prepare) (www.nc3rs.org.uk/arrive-guidelines). Importantly, we pay specific attention to sample sizes. The critical question in every animal experiment is the primary outcomes measure. We use this to ensure that animal studies are powered sufficiently to address to the scientific question. Examples include the differences required to power survival studies versus those designed to generate biological data including RNA sequencing and flow cytometry. Where possible, experiments will provide data that address multiple end points, limiting the total number of experiments/mice required. We use pilot studies for survival experiments in order to reliably estimate effect size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have named licence holders within the group who manage breeding colonies to ensure efficient breeding. We also maintain a prospective colony management database that is accessible by all researchers involved in the breeding program. Regular meetings will be held to discuss scientific requirements and any breeding issues. We use pilot studies for survival experiments in order to reliably estimate effect size. We always ensure that material generated from these pilot studies is used appropriately and never wasted. We also regularly discuss planned animal experiments within the group as well as with collaborators in order to ensure that all therapy groups are covered and that all biological material is used. This has already reduced the total number of experiments undertaken within our group.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal models and methods used in this project include genetically altered animals and procedures to induce and treat tumours. The genetic alterations we are proposing to use mimic the early changes seen in humans with pancreatic cancer before the identification of tumours. These alterations mean that animals and their tissues provide scientific information prior to the development of tumours. This minimises the harm whilst maintaining scientific output. We have also optimised a protocol for the development and treatment of pancreas tumours that are directly injected into the abdomen. By refining our imaging modalities using ultrasound and contrast enhanced CT imaging, we are able to detect and treat tumours at very early stages. This means that we are able to generate useful scientific information before the tumours become large and symptomatic. We have dedicated time and training to ensure that the procedures used to generate these tumours cause the least distress and are associated with very few complications. These imaging refinements have been introduced as a result of close collaboration with a group of researchers based at another location within the UK. By sharing data and experiences we have been able to reduce duplicity and refine our approaches more swiftly.

Why can’t you use animals that are less sentient?

We use animals at the earliest possible stages for procedural techniques, where we are only limited by weaning and the acquisition of an adult immune system. This immune system closely resembles the human immune system. Lower sentient animals such as worms, fruit flies and fish do not have an immune system that recapitulates the components of a human immune system. For the genetically altered animals, we gather scientific data and material at the earliest possible stages in order to prevent distress associated with tumour development. The mouse genome overlaps significantly with the human genome. This permits valid interpretation of findings from genetically altered models that can be translated to humans. We need to monitor the response to treatments by measuring the size of tumours over a period of time. For this reason we cannot perform the experiments under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Only healthy animals will be used for breeding. For procedures, we will inject into the tail of the pancreas as this results in less aggressive tumours and requires a smaller incision reducing the impact of surgery. Inhalation anaesthesia will be used when needed as this is less invasive and easier to regulate. Specialised radiation platforms will be used to ensure tumour specific targeting and avoid unwanted side effects. We provide the animals with non-medicated jelly in the days preceding surgery. This is then replaced with medicated jelly (analgesia) on the day of and days following surgery. Mice are observed regularly on the day of and days following surgery and if any distress/pain is observed then additional analgesia is provided. If this pain is not alleviated with additional analgesia then the animal is humanely killed. We also provide moist mash on the floor of the cages in order to reduce the need for animals to reach up for feeding. In the immediate post operative period, mice are housed on heat mats in order to maintain their body temperature. When needed additional peri-operative fluids are provided to maintain hydration. For animals with a genetic predisposition to tumours, regular monitoring will take place. This will include monitoring for papillomas and swollen abdomens. If either of these symptoms interfere with the normal activity of the animal (e.g. eating/passing urine or faeces) they will be humanely killed. Currently, the KPC/KC model results in a phenotype highly representative of the human disease pattern. It has significant advantages over transplantable (subcutaneous/orthotopic) models. It has been demonstrated that therapies that show promise in the transplantable models are not reproducible in the KPC model. This is critical in ensuring that only effective treatments are translated into clinical trials. At the present time, there are no widely available alternative models that avoid the development of papillomas in the mice. However our group works closely with other groups that specialise in the development of novel mouse models, specifically pancreatic cancer. As this work evolves, if more refined models become available, we would adopt these as soon as possible in order to refine our approach.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We refer to the following organisation for information: www.nc3rs.org.uk

https://norecopa.no https://www.lasa.co.uk

In addition, we regularly review the literature in order to identify any refinements published by other groups (in our field of work). We also attend meetings and liaise with other research groups to discuss and share best practises for our surgical and imaging techniques. This has already resulted in refinements within our institution.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

In addition to liaising with our local veterinary team, NC3Rs regional manager, named information officer and animal welfare officer, we refer to the following organisation for information:

www.nc3rs.org.uk https://science.rspca.org.uk

We also attend the institutions internal NC3R meetings.

105. Increasing the Efficiency of Nutrient Utilisation in Dairy Production Systems.

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

dairy cow, feed efficiency, methane emissions, feed processing, environmental effects

Animal types	Life stages
Cattle	juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this work is to understand the effects of basal diets, feed processing and feed additives on the efficiency of utilisation of feeds, increasing the proportion of nutrients directed to milk and reducing wasteful and potentially harmful excretions of methane, faeces and urine. We will also develop commercially applicable methods to

estimate feed efficiency and excretion of waste products such as faeces, urine and methane - based on things like milk analysis and estimates of activities such as eating and rumination through cow collars.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In order to meet growing global demand for milk whilst reducing the environmental and climate warming effects, we need to develop new feed processing methods and feeding strategies to increase feed conversion efficiency. The allied objective of developing minimally intrusive tools for monitoring feed efficiency and its components is another important element for increasing feed efficiency as the ability to closely monitor nutrition of individuals on farm is central to more efficient feeding strategies. Feed efficiency is important for both economic and environmental aspects of dairy sustainability since it affects both the yield of saleable product and the amounts of potentially harmful waste emissions.

Ruminant production is under continued political and social pressure to reduce greenhouse gas (GHG) outputs, since cattle and sheep contribute to 66% of total UK agricultural emissions (BEIS, 2018). This project will contribute to government targets for reduction in UK emissions of greenhouse gases (net zero by 2045), through targeting direct reduction of methane emissions as well as through reduced feed use resulting from more efficient conversion of feed into products.

What outputs do you think you will see at the end of this project?

Dairy cow production data (e.g. feed intakes, milk yields, milk composition, outputs of methane, faeces and urine) providing information and understanding of the effects (and mechanisms for effects) of different feeding strategies, feed formulations and feed additives.

Basis for new proxies (e.g. new analysis of milk samples) that can be used to estimate feed efficiency and emissions.

Applications of animal sensors (such as pedometers and activity sensing cow collars) to monitor feed efficiency, emissions and factors affecting them.

Who or what will benefit from these outputs, and how?

The beneficiaries of this work will include academic scientists, animal nutrition and supplement companies, animal breeding companies, farmers, governments and other

policy-makers, climate scientists, environmentalists, animal health and welfare groups and the general public.

Academic Impact:

Key new insights will be about combinations of feed additives/strategies, interactions of feed additives/feeding strategies with cow genetics and cow state (e.g. stage of lactation, parity and body reserves)

Food security – UK and globally:

Meeting the food demands of the growing world population, whilst reducing the effects of agriculture on climate change is one of the greatest challenges that agriculture has faced to date. This research project will provide new feeding strategies, new additive approaches and monitoring technologies to achieve the necessary increases in feed efficiency and reduction in emissions.

Reduced production costs and improved efficiency will enhance the sustainability and reputation of UK dairy production at a time (post-Brexit) when the industry has opportunities to develop new export markets based on high-quality products with high environmental credentials.

Sustainable dairy production will provide long-term stable rural employment, supporting regional growth and communities.

UK dairy producers:

The increased efficiency and reduced emissions will provide a significant market advantage as the consumer demand for sustainably produced food with low environmental impact is increasing rapidly.

Consumers:

The demand for food with low environmental impact is increasing, the development of feeding strategies and aligned management tools will produce low carbon dairy products and increase the acceptability of UK produce for local and foreign consumers.

Dairy processors and supermarkets:

We work closely with dairy processors in our region and their market research highlights the increasing consumer demand for more environmentally friendly products.

Industry:

Our extensive range of industry partners (feed providers, supplement manufacturers, dairy engineering companies and technology developers) will ensure that outcomes of the research can be implemented quickly.

How will you look to maximise the outputs of this work?

Scientific outputs of the work will be disseminated through publications in peer reviewed journals, national/international conferences and through social media and dedicated web-pages. Results will be embedded into undergraduate degree programmes and doctoral-training programmes.

The work is supported by a large network of commercial industry partners, who will support in disseminating project outputs and developed solutions and knowledge to the dairy industry. Our dairy processing partners and own consultancy business are well placed to drive change in practice.

Dissemination of the work will be across the agricultural community and wider network of businesses connected to each collaborating industry partner. This will include trade events, farmer-workshops, press and online channels and the wider network connected to collaborating industry partners.

Species and numbers of animals expected to be used

- Cattle: 500 dairy cattle (juveniles or adults)

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We need to work with commercially relevant dairy cattle, both to quantify and understand the effects of feeding/additive strategies being developed – and to provide credible evidence to potential users and stakeholders who will drive adoption.

Dairy production systems are under significant and continued political and public pressures to reduce their environmental impact and optimise feed use efficiency. It is important to investigate methods of alleviating this impact using dairy cattle. Previous work has demonstrated the potential for modifying the diet of ruminants to mitigate methane and/or improve feed efficiency. There is growing evidence that novel feedstuffs and feed additives can significantly reduce methane production, whilst tactical use of supplements can increase feed utilisation.

It is not possible to develop new proxy tools for methane and feed efficiency without working with the relevant farm animal species under normal management conditions. Once developed, these tools will allow routine and non-intrusive monitoring of many animals on commercial farms.

Typically, what will be done to an animal used in your project?

Animals may be enrolled on this licence at any stage of life and may remain on the licence for several years, during which time they may be managed on standard farm diets for extended periods without regulated measurements, whilst at other times they may be offered experimental diets and undergoing regulated measurements. This may be different diets or additives offered for extended periods (or all) of a lactation (in a continuous design) or a series of feeding strategies/diets/additives being compared simultaneously in a changeover-design study (typically with period of 3-5 weeks repeated 3-5 times). During this time, animals may experience some or all of the regulated procedures (all are optional); they will then return to standard farm diets with no regulated procedures and may move on to other test strategies/diets/additives in the same or subsequent lactation. The objectives of this work mean that we may alter diet composition beyond the normal range of commercial feeding practices (but not to extremes that could not be used commercially) and use unlicensed feed additives. Animals may have an electronic ear tag applied for identification by monitoring equipment, including animal mounted sensors such as pedometers, collars and rumen boluses. Samples for monitoring effects of the diets/additives on rumen function and/or development of new proxies to predict feed efficiency and emissions may include rumen fluid, faeces or blood.

What are the expected impacts and/or adverse effects for the animals during your project?

It is essential for our research objectives that the animals perform well, according to their genetic potential, and all procedures in this project are mild. Animals will be adapted to dietary treatments gradually and they are only likely to cause mild, transient digestive upsets. Sampling procedures are minimally intrusive, whilst tube sampling of rumen fluid is non-surgical and causes only transient discomfort.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We anticipate that the severity of all procedures used in this project will be mild. The most severe procedures used in this study are blood and rumen sampling where only short-term transient discomfort is anticipated.

What will happen to animals at the end of this project?

- Rehomed
- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is not possible to evaluate between-animal variation in feed conversion efficiency and methane emissions, nor to test the use of new precision livestock farming tools for the monitoring and management of livestock without working with the relevant farm animal species under normal management conditions.

Which non-animal alternatives did you consider for use in this project?

Whilst some of the work on feed additives builds on initial screening using in vitro systems, which may use either enzymes or rumen fluid, it is not possible to evaluate between-animal variation or novel markers, proxies or sensors to monitor animal feeding, performance, feed efficiency or methane emissions without using them with the relevant farm animal species under normal management conditions.

Why were they not suitable?

Whilst in vitro methods are important for screening diets and additives (allowing us to exclude unpromising options), quantifying and demonstrating responses means that work must be conducted in farm animals, under commercial farm conditions. Adaptation in the system (e.g. the rumen microbiome or contribution of mobilised body reserves) means that some promising options from in vitro work do not work when evaluated in animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We estimate enrolling up to 3 groups of 40 cows in the first year and that with animals remaining on the project into subsequent lactations (with intervening non-recording periods) we may enrol fewer cows in subsequent years - so 500 dairy cattle in total.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For each phase of measurements, experimental designs and methods of analysis have been or will be discussed with expert statistical support. The design of individual experiments will maximize the information obtained from the minimum resource and will draw on our own experience of running similar experiments to optimise the number of animals required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where appropriate we will use in vitro screening of potential treatments, use pilot studies to provide baseline data or information to inform the optimal size of treatment groups. In some areas of work we are able to share samples and data with collaborators - for example in the work on N isotopic fractionation as a proxy for feed conversion efficiency we have participated in two large international meta analyses that are now published in refereed journals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use breeds and crosses of dairy animal that are relevant to dairy farmers in our region – this currently means Holstein-Friesians, but we are considering incorporation of cross-breeding to increase heterosis and health and welfare benefits. All sampling procedures are mild and designed to be minimally intrusive because we need animals to be performing according to their genetic potential and as per normal commercial practice. Equipment and methods for recording feed intakes (Insentec HOKO feeders), milk production and composition (milk meters and samplers approved by the International Committee on Animal Recording (ICAR) and methane emissions (GreenFeed system) are all internationally recognised methods that are well accepted in the scientific literature.

Why can’t you use animals that are less sentient?

It is not possible to use immature, less sentient or terminally anaesthetised animals because we need to test feeding strategies and develop and test proxies and measurement tools that are commercially applicable to farming environments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Many of the techniques have been used at our facility for many years and we have experienced technicians working closely with our vets to refine procedures. Developments are in the areas of monitoring cow comfort and activity, attention to cow beds, and systems and equipment to minimise noise and stress during handling and sampling.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our research always consider accepted norms for refereed publications in the discipline in terms of methods and duration of recording. Some of this is detailed in international guidelines for animal phenotyping. As members of a large EU Infrastructures project we have contributed to some chapters, and edited, an online handbook providing guidance for many of the specialist techniques used in farm animal research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are actively involved in (and collaborate nationally and internationally on) research to develop proxies and animal sensor technologies that are minimally intrusive and which could easily be applied on commercial farms - so we keep a close eye on 3Rs developments. We will also monitor NC3Rs website and other material from outside the cattle research area to identify potential new 3Rs approaches.

106. Defining the Mechanisms that Influence the Generation and Progression of Blood Cell Cancers

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

chronic leukaemia, cancer, therapy, surrounding tumour environment, cancer stem cells

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to gain a deeper knowledge of the mechanisms that regulate the development of normal blood cells and blood cancers. In this way we will identify weaknesses in blood cancer cells that can be exploited to generate new treatments for disease. This knowledge will enable us to enhance current treatments options of patients with the development of novel drugs and/or novel drug combinations.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chronic leukaemias are diagnosed in ~4,600 people in the UK/year and are responsible for ~1,200 deaths/year, with chronic lymphocytic leukaemia (CLL) being the most common leukaemia in the Western world. Blood cancer patients have benefitted from the introduction of more targeted, less toxic treatments (so called targeted therapies) in the last decade, resulting in a significantly increased survival and an elevation in quality of life. However these targeted therapies are not suitable for all patients, either due to the toxic nature of the drugs or because the cancer becomes resistant to the drug and progresses, therefore requiring different treatments. For these reasons, it is important to understand the mechanisms by which the leukaemia becomes resistant, and find alternative, more effective drugs that can be used as treatments for these blood cancers.

What outputs do you think you will see at the end of this project?

This project aims to find weaknesses in blood cancer cells that help with the discovery of new ways to treat blood cancer. These results will be made available to doctors and scientists by publishing in high- quality cancer and haematology (blood cell) journals, and sharing unpublished data at conferences in the form of abstracts and poster/oral presentations.

Who or what will benefit from these outputs, and how?

The results of this project will benefit patients with blood cancer by aiding in the development of kinder and more effective treatments, and discovering new markers (biomarkers) to help clinicians identify those patients that will respond to specific therapies. Identifying new drug targets will permit the delivery of clinical trials to patients. This approach will focus targeted therapies towards appropriate patient populations, establishing a personalised care pathway, and deliver quality adjusted life years for patients, through the reduction of disease burden.

Our research may also benefit the NHS by reducing the costs of treatment, and enabling patients to have treatments delivered as out-patients rather than requiring extended stays in hospital.

How will you look to maximise the outputs of this work?

The results of this work will be discussed at scientific meetings with other experts in the field. Any scientific data generated from the experiments will be made available to other scientists after they have been published. We will collaborate with other researchers and will share our knowledge and expertise as much as possible.

Species and numbers of animals expected to be used

- Mice: 20,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our previous work has shown that if we give mice leukaemia, either by transplanting mice with mouse cells engineered to replicate chronic leukaemia, or patient cells isolated from leukaemia patients, these cells grow and spread around the body in a very similar way to patients. Using these mice with leukaemia, we have already discovered important markers on the surface of leukaemia cells that can help differentiate normal cells from leukaemic cells, thus providing an improved approach to determine disease levels in patients. Furthermore we have used these mice to identify important genes that might be responsible for keeping the leukaemia cells alive, and further demonstrated that these genes are also important in patients with leukaemia. This means that mice are very good "model systems" for studying leukaemia and how it will respond to treatment in different organs in the body. We typically use young mice (usually 6-8 weeks old) in these experiments, but as chronic leukaemia is more generally a disease of the elderly, we sometimes transplant these mice with blood cells isolated from older (up to 20 months old) mice. In addition, in some experiments we will use older (up to 15 months old) mice to allow us to look at the impact of aging on leukaemia development. Understanding these differences will allow us to develop more personalised treatments for patients.

Typically, what will be done to an animal used in your project?

The project covers a broad range of techniques, with around 50% of mice used for breeding and maintenance purposes, generating the mice required for experimental purposes. Of the remaining 50% of mice, 5% will be used to generate tissue, 5% will be aged or placed on special diets, and the remaining 40% will be transplanted with leukaemia cells.

For transplants, 50% of the time we will use mice that are immunodeficient (mice that are unable to fight disease) injected with human leukaemia cells (called xenograft experiments) and 50% of the time we will use genetically altered mice that develop leukaemia due to the genetic mutations they carry (called genetically altered (GA) mice).

For xenograft experiments, the immunodeficient mice may receive a non-lethal dose of irradiation (70% of a lethal dose) one day prior to being injected with human leukaemia cells into a vein in their tail - this is relatively painless and quick. The irradiation step makes room in the bone marrow of the mice for the transplanted cells, improving the chances of their growth (engraftment) similar to the conditioning stages that patients

receive prior to a bone marrow transplant in the clinic. In some cases (10% of xenografts) the mice will be put under anaesthetic to allow us to inject the leukaemia cells directly into the bone marrow using a fine needle inserted into the leg bone - they will be given painkillers to relieve any discomfort which usually lasts less than a day. In both cases, the leukaemia cells grow in the mice and spread around the body. The mice will have regular health checks (at least twice a week) and may undergo blood tests and possibly scans/imaging to look at how fast the leukaemia is growing. These tests are usually done once a week. Depending on the leukaemia progression, the mice will start to appear unwell between 6-12 weeks. At this point we will terminate the experiment to prevent suffering.

For our GA mice experiments, mice may receive a non-lethal dose of irradiation (50% of a lethal dose) one day prior to being injected with mouse GA leukaemia cells into a vein in their tail. The mice will have regular health checks (at least twice a week) and may undergo blood tests and possibly scans/imaging to look at how fast the leukaemia is growing. These tests are usually done once a week. After 4-10 weeks the mice usually start to appear unwell. At this point we will terminate the experiment to prevent suffering.

For 80% of all transplanted mice, we will try out new treatments to see if they can get rid of the leukaemia or slow its growth. Treatments can be given by injection, by mouth or added to drinking water or food and will typically last up to 4 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?
Irradiation causes:

1. Weight loss, diarrhoea at the beginning of week 2, which resolves by around the end of week 3 post irradiation.

2. Increased risk of Infection

3. Tooth growth in specific GA mouse strains

Leukaemia causes:

1. Low blood counts (anaemia) - causing reduced activity and tiredness/lethargy - this lasts 1-2 weeks on average

2. Increased risk of infection

3. Weight loss - this is seen in the last 1-2 weeks of the illness

4. Small tumour formation can occur throughout the body (e.g in lymph nodes, liver and spleen) in the later stages of the disease.

Treatments may also cause side-effects such as loss of appetite, diarrhoea or fur loss

Injection of leukaemia cells directly into the bone marrow can cause discomfort which usually lasts less than a day and is helped by painkillers.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild severity (causing no more than momentary discomfort) - 50%

Moderate severity (discomfort lasting no longer than 72 hours on any occasion) - 50%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To develop new drugs we need to understand how leukaemia cells behave in different environments within the body. We are currently unable to recreate these complex biological environments (comprising multiple different cell types, nutrients and blood supply) in test-tubes or tissue culture flasks. By using mouse models, we can not only identify molecules responsible for leukaemia maintenance or therapy resistance but also modify the leukaemia cells using genetic approaches and/or test new drugs to see if this can be overcome. These are essential to develop new clinic-ready therapies for patients with leukaemia and other blood cancers.

Which non-animal alternatives did you consider for use in this project?

All preliminary work (such as testing whether drugs kill leukaemia cells and what dose to use) will be done in the laboratory with cells grown in culture, enabling us to rationalise which experiments to carry forward into mice. Since we need to study cells taken directly from different sites around the body we have two alternatives - animal models or patient samples taken from the lymph nodes and peripheral blood. We have permission to use patient samples and use these when they can address our research question.

Why were they not suitable?

Cell culture: Our central aim is to understand how leukaemic cells behave in different environments within the body and how treatments are affected by these different

environments. We are currently unable to recreate these complex biological environments (comprising multiple different cell types, supporting matrix, nutrients and blood supply) accurately in test-tubes or tissue culture flasks.

Patient samples: While we do use patient samples, patient material is often limited and generally does not survive or grow once outside the body. Importantly, we cannot test experimental therapies directly in humans and cannot genetically alter leukaemia cells and inject them into patients.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

As active researchers we are routinely exposed to new experimental approaches and will incorporate them into our future research programme if they prove to be as reliable and robust as the proposed animal experiments. In this way we will endeavour to reduce the number of mice that are proposed for use in this project licence.

I have used statistical methods to determine that I will require around 5-7 mice per experimental group and typically experiments will compare 3 or 4 groups. Based on our current funded work and planned future work we will require 4,000 mice per year to perform our research.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I have used both expertise from statisticians and the NC3Rs' Experimental Design Assistant. We have considerable experience in carrying out the protocols described and will gain the maximal amount of scientific information from an appropriate number of mice, using serial non-invasive imaging and/or blood sampling of cells from the mouse during disease development and/or treatment to gain a detailed picture of the blood cancer developing in the mouse. Therefore data will not just be generated at the endpoint, but throughout the course of the experiment.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

I will employ the most efficient breeding strategy at all times. Ongoing monitoring of a given cohort (mouse group) allows experiments to be stopped as soon as there is enough data (information), thus minimising suffering whilst obtaining meaningful and publishable

results. For any new treatments we will always treat a small pilot cohort to estimate the effect size before performing the full experiment. When possible control mouse cohorts can be shared across studies, to reduce mouse numbers required for individual studies. We always maximise all the information we get from each mouse.

During the experiment blood sampling and non-invasive imaging techniques will be carried out to obtain as much data from a smaller number of animals. At the end of the experiment we will analyse as many tissues as possible per mouse by harvesting spleen, bone marrow, lymph nodes and blood and sharing this tissue between different members of our research group working on different projects.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use a variety of mouse models in this project. Some models have been developed to be more susceptible to developing leukaemia and therefore we can study them without having to expose the mice to harmful irradiation, although this is not always possible. Other models have markers that are identifiable by imaging of the mice, thus allowing more straightforward investigation of processes within the organ(s) of interest without the need for additional invasive procedures. All these models are developed to cause the least pain, suffering or distress possible while providing us with valuable data on the disease of interest. Furthermore, we have introduced detailed methods of recording the health status of animals undergoing procedures which enables changes in their well being to be detected earlier in the disease process.

Why can’t you use animals that are less sentient?

Mice are still sufficiently closely related to human beings to adequately represent the human condition, whereas other model organisms more distantly related would not replicate the disease sufficiently (on a genetic and disease characteristics level).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal monitoring, post-operative care, and pain management are an important part of all the procedures within the project. The need to minimise suffering is always considered

when planning experiments and we routinely revise our experiments to reduce animal suffering. During this licence we plan to refine the way we promote the engraftment of blood cancer cells into mice, and instead of using irradiation which can cause side effects in the gut, we will develop a more targeted approach thus improving the welfare of the animals. Of note, none of our experiments exceed a moderate severity level.

All mice on experimental procedure will be frequently monitored (a minimum of 2 x weekly) and humanely culled when exhibiting signs of altered health status and/or tumour burden or another specified endpoint is reached. We have developed a stringent distress scoring system that allows an immediate identification of mice with adverse effects. All researchers working on this project will undergo specific training in monitoring leukaemia development and health status in our models. We will refer to the literature to identify any likely adverse effects of a new agent and when a genetic cohort is given a treatment for the first time, pilot studies will be carried out and closely monitored before extending to a larger cohort. All animals are housed in a dedicated facility proactive with environmental enrichment and the use of anaesthesia and analgesia under guidance from the named vet is routine practice. To minimise infections, immunodeficient mice will be housed in barrier caging under sterile conditions and handled in sterile cabinet that protects the mouse from infections. Post- mortems are carried out to investigate any unexpected deaths.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For all studies we will refer to the Guidelines for the welfare and use of animals in cancer research (Workman et al, 2010) and ensure best working practice. We consult the NC3Rs guidelines and monitor refinement when practice advances are published (https://www.nc3rs.org.uk/).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All our work observes government guidelines and we adopt new guidance, from the Home Office or our local AWERB, as soon as these are recommended. We are also actively involved in our establishments 3Rs Day, and there are a number of Culture of Care events organised throughout the year to ensure everyone stays up to date with current guidelines. I also routinely check the following website: https://www.nc3rs.org.uk/ monthly for updates and will address any updates at our weekly lab meeting and disseminate this information to all personal licence holders working on the project.

107. Production and Maintenance of Genetically Modified Zebrafish

Project duration

5 years 0 months

Project purpose

- Basic research

Translational or applied research with one of the following aims:

(i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Zebrafish, breeding, husbandry, genetically modified, genetics

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The purpose of this project is to establish a service licence to create, breed and maintain zebrafish, most of which will be genetically modified. Studying these animals and the embryos they produce will expand our understanding of how genes contribute to various human disorders.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Zebrafish are a commonly used animal system to study gene function and how these genes are altered in human disease. As zebrafish genes, organs and tissues share many similarities with humans, what we learn in zebrafish helps our understanding of how the genes work in humans. Many of the changes to genes which cause thousands of different human disorders in adults and in children are currently poorly understood. By studying similar genetic changes in zebrafish we can begin to answer which genes are important or not in human disorders.

Genetic changes that we will study under this licence are those related to skin cancer, eye disorders, alcohol sensitivities which increase the risk of certain cancers, jaw deformities and others.

This licence will allow skilled, experienced animal technicians to provide full husbandry care and the highest welfare standards for genetically modified zebrafish to be studied by researchers at our institute. These experiments will allow the study of mechanisms of development and disease to inform our understanding and treatment of human conditions. These researchers will receive experimental training and support from a full time staff member who is highly qualified in animal welfare.

Our dedicated zebrafish facility and staff can support in depth, and up to date, knowledge of animal welfare and husbandry which would be difficult for individual labs to maintain.

What outputs do you think you will see at the end of this project?

This licence exists to support research into the fundamental mechanisms of human disorders.

In the short term: Generating new genetically modified zebrafish, which are subsequently available to all researchers as tools for investigating human genetic disorders.

In the longer term: Helping to understand disorders and supporting the development of methods to manage and treat human disease.

The benefits of this service licence will be supporting research into the fundamental mechanisms of human biology and disease. This is worthwhile because it provides the basis for our understanding of human and animal life, as well as potential therapies.

The scientific output from the work performed under this licence has been and will continue to be published in peer reviewed, high impact, leading journals.

Who or what will benefit from these outputs, and how?

In the longer term, the general public and people suffering from genetic disorders may benefit from medical advances informed by this work. Human genetic disorders are varied

and complex and will impact almost every human being directly or indirectly through relatives and friends. The real costs are, realistically, incalculable but enormous. Understanding the underlying genetics in a model vertebrate, the zebrafish, is a major step towards developing treatments for these disorders.

Scientific user support and animal welfare

In the shorter term, researchers at our institute will benefit from the skilled, centralised support to provide healthy animals with the highest welfare standards for their research, leading to more reliable data and peer reviewed publications.

Having a central service licence in conjunction with dedicated facility staff provides benefits to welfare and scientific support simultaneously. The welfare benefits will be discussed in more detail in the later 3Rs section and are only mentioned here in their connection with benefits to facility users.

A central service licence, in conjunction with dedicated facility staff, will allow unified support of a critical mass of zebrafish researchers and available zebrafish lines. A benefit of this will be that new researchers or researchers who are new to zebrafish will have experienced support for them to efficiently and ethically make use of zebrafish. Furthermore, supporting and encouraging the use of a classically ‘lower’ vertebrate can reduce the use of mammalian models as well as other implementations of the 3Rs which are outlined in the later section.

The central support licence will ensure that decisions and practices are uniform and of the same high standard for all zebrafish experiments and expertise is readily available and shared, rather than individual labs having to arrive at their own systems for generating and maintaining zebrafish lines.

In addition to economy of scale and reduced need for oversight of multiple individual labs, a further benefit of centralisation of the fish facility is the ability to carry out long range, large projects, coordinated under a single licence. e.g. The creation of a cryopreserved sperm repository to safeguard lines. This reduces the risk that a line might be lost and would have to be recreated and reduces the number of animals needed to maintain lines.

The wider zebrafish research community will also benefit from the work performed under this licence. The new lines that are created under this licence will be shared widely as appropriate. Welfare improvements and advances which support the 3Rs will be disseminated through local meetings as well as nationally and internationally through attendance of conferences.

How will you look to maximise the outputs of this work?

As the main purpose of this licence is supporting the use and creation of genetically modified zebrafish, we will maximise the utility of these lines by sharing them widely, as

appropriate, to researchers around the world. This is supported by the facility team who have experience with embryo and animal export.

Freezing sperm for each new line created ensures these lines will be available for future use, within our institute and elsewhere.

Researchers at our institute are encouraged to submit findings to bioRxiv (https://www.biorxiv.org/) which will make their findings available to everyone, with or without publication in a peer reviewed journal.

Knowledge gained and improvements devised to husbandry and welfare through caring for the animals on this PPL will be shared with the local, national and international zebrafish community through regular meetings organised by researchers, facility managers and zebrafish societies (e.g. Zebrafish Husbandry Association weekly meetings).

Ongoing improvements to protocols on this licence will be used to inform other licences within the university, present and future, through the university vets and the contributions of the facility manager to the Animal Welfare Ethical Review Board (which offers feedback to all proposed licences).

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 28,500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The zebrafish is one of the fastest growing model organisms in terms of exponentially growing numbers of research articles, funded projects and new discoveries associated with the use of zebrafish for studying development, human diseases and screening for new drugs. Thanks to the development of novel technologies, the range of zebrafish research is constantly expanding with new tools synergistically enhancing traditional techniques.

The zebrafish is a unique vertebrate model system that can provide valuable insight into human genetics and biology due to multiple factors:

- The zebrafish is a small vertebrate whose genome has been sequenced.

- The genomes of zebrafish and humans share significant similarity. This similarity can be found at the levels of cells, tissues and organs.

- Rapid embryonic development occurs such that by 48 hours post fertilisation all major organs have developed and are functional. This development naturally takes place externally so no invasive procedures are performed on the parent animals.

- Due to this rapid development, many studies can be carried out on the majority of organs before the embryo is classified as a protected animal thus greatly contributing to Reduction.

- Even before 5 days old, the larvae exhibit stereotyped behaviours which can be studied.

- External development and transparency of the embryo allows longitudinal studies (multiple time points from one animal) which, in other models, would require animals to be sacrificed for each time point.

- The transparency of the embryo synergises with the vast genetic toolbox of the zebrafish to allow expression patterns to be followed in vivo.

- Embryos are freely permeable to soluble drugs and vital dye staining,

- Large clutch sizes of large (1mm diameter), robust eggs are laid daily on cue (light) resulting in synchronised cell development of hundreds of embryos.

- These robust embryos are accessible and readily amenable to well-established genetic manipulation, with no harm to the mother.

- A wealth of knowledge (databases and resources) already exists for zebrafish (e.g. www.zfin.org; www.ensembl.org/Danio_rerio/)

- For a vertebrate housing facility, required space and costs are modest, thus supporting cost effective research.

For these reasons, use of zebrafish as a model organism in the UK is rapidly increasing and it is likely that more labs will want to use them in the coming years. With a facility licence in place, new researchers, or researchers who are new to zebrafish, will have the support they need to efficiently and ethically make use of zebrafish. Furthermore, supporting and encouraging the use of a classically ‘lower’ vertebrate can reduce the use of mammalian models.

Our unit has a focus on genetics. Genetics has an impact on almost all aspects of human biology and, increasingly, medicine. While the study of human populations and patients can inform us about the role of genes, a full understanding of gene function and how these genes interact with environmental factors and drugs still relies on the study of model organisms.

Our unit mission is to identify the molecular and cellular mechanisms underlying normal human development, maintenance and disease, and to translate these findings for clinical benefit. A large part of this work is achieved by studying the function of genes in model systems, including in vitro systems, cell based systems and animals. As a vertebrate, zebrafish provide a relatively easy, inexpensive and valuable system to study the mechanisms of human genetic diseases and treatments. There is a need for our scientists to be able to use the zebrafish system to address specific experimental questions in their research.

The vast majority of work will be done with embryonic/larval zebrafish which are less than 5 days post fertilisation. At this stage they do not have the status of protected animals as their capacity to suffer is less than that of more developed animals.

When creating new genetic modifications, in the vast majority of cases the alterations will made at the 1 cell stage (immediately after fertilisation of the egg). At this stage the fertilised egg is incapable of suffering.

Typically, what will be done to an animal used in your project?

The majority of animals on this licence will not have any procedures performed on them. They will be kept in our state of the art aquariums to produce fertilised eggs by natural spawning which will be used in experiments.

A minority of animals will have a small piece of fin removed by scalpel under anaesthetic which will grow back fully within 2 weeks.

A very small minority of animals will be gently squeezed with a finger tip under anaesthetic to release sperm or eggs for long term storage or artificial fertilisation.

A very small minority of animals may be non-invasively imaged by anaesthetising them for brief photographing under a microscope or other imaging modalities.

A very small minority of animals may be treated to activate their modified genes by briefly heating their water by about 10C or immersing them in a dilute concentration of a commonly used drug which is not harmful at these concentrations.

What are the expected impacts and/or adverse effects for the animals during your project?

Most fish will not experience any adverse effects from the changes made to their genes. As some of the genes changed are related to human disorders, a minority of fish may have problems which relate to the function of the gene. This depends on the gene in question e.g. fish with genetic changes related to skin cancer will have a higher rate of developing skin cancer, while fish with genetic changes related to human eye conditions may have a higher rate of eye problems.

Most fish will experience no adverse effects.

A minority will experience minor, temporary discomfort, such as cutting off a small piece of a fin under anaesthetic (which then grows back naturally).

A very small minority (likely to be less than 1%) will experience medium adverse effects, such as developing skin cancer. Theoretically, but not yet observed, specific mutant lines may develop eye defects, jaw defects, cilia defects, dwarfism or nervous system defects as the genes being studied are expressed in these tissues. Any zebrafish which is approaching the limit of allowed severity will be humanely euthanised before the limit is exceeded.

All zebrafish will be humanely euthanised at the end of the experiment or when they reach their maximum healthy lifespan.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Based on previous experience, 85% of animals will experience subthreshold severity. 14% will experience mild severity. 1% will experience moderate severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals are required because there is no non-animal system that can provide us with the insight into how these genes work within the context of a living vertebrate system.

We try to only use zebrafish after first learning as much as we can using computer models, cells in dishes and simpler animals.

Most of the experiments will be carried out on zebrafish larvae that are less than 5 days old and not considered developed enough to be protected animals.

Which non-animal alternatives did you consider for use in this project?

This service licence supports a range of projects which also use computer models, cells in dishes and organoid cultures (mixtures of cells grown in dishes which partially mimic organs).

Why were they not suitable?

Most genetic disorders are dependent on complex interactions between genes, cells, tissues and organs in living animals. This can only be achieved to a limited degree with organoid cultures and to no real extent in cell cultures.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We keep a database of all animal use which provides us with historical and current usage numbers to base projections on. The facility manager is aware of project proposals and animal needs associated with them for each lab. Costed grant applications also indicate future demand. We have already held similar service licences for over a decade and use this experience to inform projections.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The individual labs that are supported by this service licence have access to our unit's statisticians and experimental design experts and are encouraged to seek their collaboration before data collection begins.

In the majority of cases, embryos are used before they reach protected status, which does not contribute to animal use numbers. The number of adult animals required to generate the embryos can be kept lower by excellent welfare standards which keep the animals at maximum fertility. Strategic planning of different phases of projects helps to avoid spikes in demand for embryo production by staggering use.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Before we make a new genetic line of zebrafish we make sure it does not already exist somewhere in the world.

We breed only the animals we will need to use or to increase or replace our breeding stocks. We keep a database to keep track of all the fish and procedures.

If a genetic line of zebrafish is not being used, we will freeze the sperm and keep it until the line is needed again.

Facility staff coordinate with researchers to establish communal animal pools, which are shared by multiple individuals, to prevent individual researchers duplicating animals unnecessarily.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Unlike most other animals used in the laboratory, zebrafish embryos are externally fertilised and are transparent during early development. This allows for imaging of cell and tissue development in a living animal without harming it.

Pain will be controlled by using anaesthesia and analgesia.

We will not keep sick fish or animals with old age related health problems.

We have a highly skilled staff that is focused on improving their skill set to refine experimental procedures and handling to reduce stress to the fish.

We will stay up to date and well informed on all animal welfare developments relevant to zebrafish and will communicate this information to everyone working with the fish.

Why can’t you use animals that are less sentient?

The vast majority of experiments performed are on embryos on the first 4 days of development before they reach protected animal status. This is already considered non or very low level of sentience.

The majority of procedures on this licence relate to the fact that the adult fish carry a genetic alteration. The majority of these animals experience no suffering from this genetic alteration that they carry.

For the minority of experiments that may cause suffering to fish, a living vertebrate animal (comparable to humans genetically and in terms of organ development) is essential to study disorders which impact humans through complex interactions within the body. Alternatives to study human genetic disorders in a different vertebrate model would be animals which are arguably more sentient (e.g. mouse) and therefore the alternatives would involve increased potential for suffering.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Unlike most other vertebrate model systems used in the laboratory, zebrafish embryos are externally fertilised and are transparent during early development. This allows for detailed visualisation of cell and tissue development in a living vertebrate animal. This also allows for longitudinal (multiple time points from one animal), non-invasive studies (pre free feeding) which in other species would require multiple animals to be culled for each time point (including the mother).

Pain will be controlled during fin-clipping by general anaesthesia and analgesia. Users will be encouraged to use, and offered training in, alternative methods of genetic material collection such as embryo fin-clip and adult surface swab.

Our local colony management database allows us to closely monitor the age of all fish and plan breeding accordingly to avoid keeping older fish while waiting on the next generation to mature, thus minimising suffering by avoiding ageing related conditions.

Adverse effects from a genetic mutation will be minimized by maintaining the fish as heterozygous (partial, rather than full mutant) lines if possible, and/or raising fish in areas of reduced daily stress and noise (e.g. darkened tank, reduced water flow, away from general traffic). Daily assessment of behaviour and mortality for each line will make it clear if any lines are suffering adverse effects.

Access to the fish facility will only be given to those who genuinely require access and pass internal training, minimising disturbances.

The facility manager is a nexus for increased coordination within the facility (having a central overview of activities in the zebrafish facility allows for coordination of activities between otherwise unconnected labs) and between other zebrafish facilities, for information exchange and to minimise duplication of lines.

Improvements in zebrafish husbandry are constantly being sought and applied, as well as the continued professional development of staff. The zebrafish staff, involved in service provision, are of a high calibre and their skills are relevant and up-to-date, ensuring minimal number of animals are required to generate new lines. The experience and focus of these dedicated staff on animal welfare is above what could reasonably be expected of individual labs.

Further benefits of having a central service licence in conjunction with dedicated facility staff will be that new researchers or researchers who are new to zebrafish will have experienced support for them to efficiently and ethically make use of zebrafish. Furthermore, supporting and encouraging the use of a classically ‘lower’ vertebrate can reduce the use of mammalian models.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The majority of the animals on this licence will not undergo experimental procedures therefore refinement largely relates to the welfare and husbandry the animals experience throughout their lives.

We take guidance from the peer reviewed guidelines of "Zebrafish: Housing and husbandry recommendations" which was published by a group of experts assembled from the 2 leading international bodies concerned with use of fish as animal models in science.

The dedicated team of experienced zebrafish technicians who care for our animals are trained to high standards of animal welfare and ethics as they have received training for personal licences, project licences and to hold the position of Named Animal Care and Welfare Officer. This training is approved by and assessed on behalf of the Home Office.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The facility manager and Named Animal Care and Welfare Officer will stay abreast of developments in animal welfare and legislation (such as by attendance of events specifically for fish facility managers and those directly involved in promoting animal welfare). The dedicated zebrafish facility manager will have a greater and constantly updated knowledge of zebrafish husbandry, welfare and disease than could reasonably be expected of group leaders. The same is true for knowledge of legislation. The facility manager is ideally placed to act as liaison to new or established researchers within the facility, disseminate any important information regarding welfare and legislation and guide how it is put it into practice.

The manager of the facility is a member of the 2 leading zebrafish focused international organisations and attends their regular interactive meetings which disseminate the latest advances in welfare and husbandry

108. Ecology of Fish and Risks from Human Activities

Project duration

5 years 0 months

Project purpose

- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Electronic tagging, Species conservation, Sharks, Marine fish, Anthropogenic risk

Animal types	Life stages
Carcharhiniformes (requiem sharks)	juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Improve knowledge of the natural history of teleosts (bony fish) and elasmobranchs (i.e. cartilaginous fish - sharks, rays and skates, hereafter “fish”) including their exposure to risks from human activities in the marine environment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Effective management of marine species requires robust data describing their distribution, movements and responses to human activity. This knowledge is invaluable in order for populations of fish to deliver important ecosystems services (e.g. nutrient cycling, food provision and ecosystem biodiversity) upon which humans and animals are dependent.

What outputs do you think you will see at the end of this project?

This project will deliver data on the natural history, ecology and anthropogenic risks experienced by marine fish. Outputs from these data will include peer-reviewed scientific research papers and advice/evidence to UK Government and Devolved Administrations (i.e. England, Scotland, Wales and Northern Ireland). Types of information produced for outputs will include: i) quantifying use of geographic areas by animals across single or multiple life stages, ii) describing animal responses to human activities (e.g. commercial and recreational fisheries, offshore engineering structures [wind farms] and habitat management), and iii) how animals interact with each other and their aquatic environments, and how this might be altered by human activity.

Who or what will benefit from these outputs, and how?

In the UK, conservation management plans are typically species or habitat-based (termed "features"). The proposed data to be collected in the lifetime of the project (<5 years) have the scope to inform management plans for these features. In particular, the risk of conflict (i.e. spatial and temporal overlap) with human activity can be evaluated to enact change in management plans that will benefit fish (e.g. improving management of breeding environments, improving management of human activity at foraging areas). In the longer term (i.e. over multiple years and decades) marine ecosystems (i.e. species and habitats "features" and the cumulative ecosystem services they provide) should benefit from the outputs if the management plans improve ecosystem health and functioning as expected.

How will you look to maximise the outputs of this work?

The outputs of the work will be maximised by sharing them directly with stakeholders through reports, peer-reviewed scientific literature and by presenting at relevant public and industrial events (e.g. stakeholder workshops, offshore wind farm and fisheries conferences). Additionally, where the project identifies 3Rs related improvements to work, these will be submitted for external peer-review with subsequent incorporation into work using the PPL amendment process. Improvements in methods (where identified) will be shared with the research community, including the NC3Rs (e.g. NC3Rs Brief Reports, Journal of Animal Biotelemetry - Case Reports).

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Fish experience a wide range of anthropogenic risks in UK territorial waters (e.g. physical and acoustic disturbance, risk of capture, exposure to chemical pollutants, loss of habitat). The project seeks to i) improve ecological knowledge of fish, and ii) understand how species-specific natural history interacts with these risks (e.g. at offshore wind farms, from commercial and recreational fisheries, and recreational boating activity). Risks encountered by these animals are likely experienced in a cumulative manner and cannot be considered in isolation (either at the level of risk or species).

Interaction with human activity will also likely vary depending on species life stage, age-related habitat preference, and nutritional and breeding condition. We will work on all life stages of the named species groups where they occur in UK territorial waters, selecting the most relevant methods based on body size and animal behaviour.

Typically, what will be done to an animal used in your project?

Animals will be subject to procedures that involve either attachment of external biologging tags, or insertion of internal biologging tags ('tagged' from hereon). The species group typically dictates which approach is most appropriate.

Study animals, following capture, are either: i) tagged on board the deck of boat, ii) tagged while restrained alongside the vessel, or iii) tagged using a propulsive darting pole.

External biologging tags archive data and then either transmit summarized data to overpassing satellites in real-time if the animal is at the sea surface or following automated detachment of the tag from the study animal after a pre-programmed attachment period. This latter type of tag floats to the sea surface and transmits summarised data to satellites or are found if washed ashore and data physically retrieved. In addition, directed searches can be made for tags that are transmitting at the sea surface which are within 20 nautical miles of land, further maximizing data returned. External biologging tags also include acoustic tags, which transmit a coded signal into the water column, and are detected and decoded by networks of underwater acoustic receivers. Networks of receivers have been deployed across coastal seas of European countries, and scientists share detections of animals they have made through the European Tracking Network (which has ~5,000 receivers to date across Europe). Acoustic tags have operational lives of up to

5 years. External biologging tags are attached to study animals in two ways: i) using darts inserted beneath the skin, which attach to the tag via a tether, or ii) using several small holes made through the main/primary (dorsal) fin on their back to allow fin mount tags to be attached. External biologging tags are manufactured to be low-profile (with respect to the body shape of the animal) and covered in compounds that prevent organisms from growing on the devices (biofouling) agents, these approaches minimise drag and limit the energetic burden of carrying the tag. Tagging is done very quickly (typically <5 minutes) to limit exposure in air. If boarded, animals are provided with flowing water over the gills to minimise gill drying and promote oxygenation. Animals' eyes are covered to minimise stress. When animals are held alongside the vessel, the vessel is orientated into the flow

of water to aid oxygenation while the animal is temporarily restrained. For very large animals that cannot be restrained tagging is achieved with a propulsive darting pole while the animal is swimming at the surface unconstrained. This process is only undertaken in calm sea conditions to ensure no injury from collision with the tagging vessel is possible and to ensure precise positioning of the tag at the desired location on the animal.

Internal biologging tags are implanted into the abdominal cavity of animals. These tags can be either acoustic tags (as described above) or archival data tags that gather information on the ambient environment (e.g. pressure) and internal animal state (e.g. core body temperature, heart rate) over the period of years. Internalised biologging tags are recovered when individuals are caught and killed (typically by commercial fisheries). Tags are labelled to aid recovery and rewards are offered to incentivise return of any discovered tags. These tags have operational lives of up to 5 years. Internal biologging tags can be implanted: i) using general anaesthesia with local analgesia (local pain relief) (for smaller species <1 metre length), followed by a surgical incision allowing insertion of the tag, sutured closed, then recovered using onboard water tanks and returned to the sea, or ii) using local anaesthesia at the site of incision (for larger species ≥1 metre length) followed by a surgical incision, with trochar (device to implant the tag)where appropriate, sutured closed, then returned to the sea and recovered using in-water towing techniques (where appropriate for the species group). Due to their large body size (≥1 metre length) some species cannot be safely subject to general anesthesia at sea as sufficiently large water tanks for post-procedure recovery are not practicable and there is a health and safety risk to staff from manual handling large animals with additional undue stress imposed on the animal.

Blood samples may also be collected from tagged fish, this process will form part of the tagging procedure and will involve blood being taken from the tail (caudal) vein. Up to three attempts will be made to obtain a single blood sample. These blood samples will be used in biochemical assays to assess animal health. A small fin clip (<1 cm2) will also be taken from some animals to provide genetic information on the origins of the animal. Body surface swabs may also be taken to determine stock of origin, sex and other biochemical analyses. The use of surface swabs as an alternate minimally invasive method to obtain high-quality genetic material (i.e. DNA) requires validation for each species group. As such, it is necessary to obtain fin clips until validation is complete. We will adopt the most refined approach that seeks to improve welfare and minimise invasive practice for each species.

What are the expected impacts and/or adverse effects for the animals during your project?

Prior work using electronic tagging of fish, has shown there is a short-term (minutes to few hours) adverse effect of tagging, particularly behavioural modification (e.g. some species swim away from the tagging site or have a higher tail beat frequency). Adverse effects are recovered quickly and expected impacts are therefore minimal. For example, published

work has shown that basking sharks (lamniformes) triple their tail beat frequency for up to 15 minutes after tagging, but then return to a baseline that they maintain for the rest of the tag deployment period (e.g. 5 days). Atlantic bluefin tuna (scombriformes) return to a steady state of swimming within 6-12 hours of release (unpublished data). Porbeagle sharks (n=5) and blue sharks (n=3) captured in recreational fisheries (unpublished data) return to a steady state within ~6 hours following tag insertion (these animals are tagged alongside a boat). Adverse effects may also be experienced by animals undergoing general anesthesia, these can include irreversible reduction in respiration and subsequent deterioration in heart and circulatory function.

In the longer-term (weeks to months) expected adverse effects are most likely linked to wound healing at the site of dart insertion, or incision into the abdominal cavity. For white sharks tagged with dorsal fin-mount tags (n=12), healed wounds associated with tagging remain permanently evident (e.g. scars) based on opportunistic re-sighting of animals showing exceptionally high levels of annual fidelity to breeding and foraging sites over several years.

There is limited opportunity and evidence with which to describe longer term adverse effects in fish because animals cannot be routinely followed up post-procedure as they have been released to the wild and are very rarely re-captured for subsequent health assessments. However, when evidence does become available it is typically from opportunistic events (e.g. capture of an animal in fisheries) or from study systems focusing on species which have repeatable behaviours enabling longitudinal studies.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severity of external biologging tagging is assessed as mild.

Severity of internal tagging is considered moderate given the requirement to surgically enter the coelomic cavity.

Teleosts:

- 71% of scombriformes (125 of 175) will experience mild severity external biologging tag attachment
- 29% of scombriformes (50 of 175) will experience moderate severity internal biologging tag insertion
- 100% of gadiformes (750) will experience moderate severity internal biologging tag insertion Elasmobranchs:

- 100% of individuals (255) will experience mild severity external biologging tag attachment

What will happen to animals at the end of this project?

- Set free
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project aims require data on the movements of tagged fish to inform management. These data can only be collected from wild animals.

Which non-animal alternatives did you consider for use in this project?

Alternative methods to catalogue natural history and behavioural responses to human activities are visual behavioural observation, sub-surface camera systems, and public sightings via photo- identification.

Why were they not suitable?

These alternate approaches often provide limited data, lack detail on spatial and temporal scale, and provide scant information about interaction with human activities often far from land. Gathering visual data on the behaviour of animals from boats or sub-surface camera systems, for example, is problematic due to the influence of the survey vessels and equipment on the animals. The named species groups do not breathe air, and so opportunities to visually sight animals are limited. There is insufficient information on these species to rely upon computer / statistical modelling approaches to address the aims of the project (indeed, the tagging data are required to inform these types of statistical models). Only electronic biologging tags can provide extended insight, location data and the potential to describe interaction with human activities across UK territorial waters.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated numbers of individuals reflect the differing aspects of natural history in these animal groups, and their expected response to perturbation by human activity. These estimated numbers have been derived from experience built delivering prior A(SP)A regulated projects and they represent a pragmatic balance between the numbers required to statistically detect potential responses, the number that can be tractably captured and the available grant funding. The number of individuals is further influenced by the cost of electronic tags, known daily capture rates, access to marine environments (e.g. constraints such as distance from port), weather conditions and the cost of days working at sea.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Sources of variation concerning animal handling (e.g. handling time, duration of aerial exposure, capture techniques) are minimised wherever possible through the use of structured sampling and handling protocols. Variation that might arise from differences in body size are either purposefully considered in the experimental design, or eliminated by selecting age/size cohorts. Experimental design made use of statistical power analyses. These analyses were primed with relevant data on the biological responses to be measured, which were sourced from relevant published or unpublished datasets, or where no usable data existed, surrogate species were considered. The applicant has a strong background in statistical analyses and has also sought advice and guidance from specialist medical health statisticians, whose work most closely resembles the types of analysis required in the project (e.g. survival analysis, dose response analyses etc.).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In addition to good experimental design, optimising the number of animals to be used will be continually informed by ongoing research across the group, by remaining appraised of scientific work published in relevant peer-reviewed scientific journals, by remaining engaged with the NC3Rs and relevant community groups focusing on animal welfare (e.g. RSPCA, LASA), by sharing our efforts and seeking relevant collaborations across the animal biologging community. We continually look to share our data with large scale meta-analyses that seek to address challenges in our field to better our scientific practice and hence minimise the number of animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will work with wild fish to better understand their natural history and how this might be influenced by human activities. The response of fish to human activity has been poorly characterised, and so wild animals need to be studied (as opposed to captive fish or digital models). Data must be collected at the individual level, and electronic tagging is the most refined approach to do this. Specific methods used will vary by named species groups, based on knowledge of their behaviour, the size of tags, safety of handling, and whether it is tractable to use general or local anaesthesia, or no anaesthesia where this is contraindicated, as animals cannot always be monitored post-surgery due to their body size. The project will investigate the efficacy of local analgesics (e.g. pain relief) for use prior to external darting. External darting is, for the majority, performed on wild fish without analgesic due to the speed in which animals must be tagged and returned to the sea (to minimise air exposure).

However, this project will gather data to quantify the effects of local analgesia on animal welfare and to identify potential improvements in the data to be collected. Wherever possible all external biologging tags are permanently and uniquely marked on the tether and the electronic tag to allow their return to the research team. This is preferred over adding additional marks with conventional identification tags (which require further punctures in the animal skin) and offers an important refinement.

Why can’t you use animals that are less sentient?

The project is dependent upon studying the responses of wild animals in their natural environments to human activity. The named species have been chosen because they are of management focus that requires underpinning data, which need to be gathered in a wild setting, and therefore it is not possible to substitute the species for less sentient animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The project will use a suite of refinements to ensure high welfare, these include:

1. Providing flowing seawater into the mouth during periods of aerial exposure (for animals that are boarded).

2. Covering the eyes to limit visual stimulus (for animals that are boarded)

3. Using low friction mats to ease movement of animals (for animals that are boarded).

4. Adopt aseptic techniques to their maximum feasible extent given the challenges of working with wild animals in the marine environment.

5. Conduct heart rate monitoring to ensure high welfare using non-invasive Doppler or minimally invasive electrocardiogram electrodes (for large animals that are boarded, where conditions allow).

6. Using local anaesthesia/analgesia at the sites of external dart placement to improve the knowledgebase on the efficacy of local analgesic use in wild biologging studies.

7. Using post-procedure in-water animal towing techniques (for larger animals), where appropriate, to ensure animals are returned to the wild in normal vigour.

8. Use of general anaesthesia for implantation of internal tags, where defined.

9. Using post-procedure recovery water tanks to monitor animals that have been subject to general anaesthesia.

10. Use of time-released post-surgery recovery cages (see picture panel; image 16) for named species subject to general anesthesia. These cages remove the opportunity of predation of tagged individuals in case of lasting (or unknown) effects of general anaesthesia. The cage automatically opens after a predetermined time (e.g. 6 hours) releasing the animal to the wild.

11. Use of cameras to record procedures for retrospective assessment of individual animal handler competency, quality control in visual health assessments, and to act as a training aid to ensure/promote transparency of procedures in difficult to access locations and ensure best practice.

12. Use of ‘What If’ scenario cards to provide rapid reference aids for project staff to determine the right course of action to maximise animal welfare in the event of emergencies (e.g. deeply hooked animals).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have gathered evidence of best practice from peer-reviewed literature, NC3Rs, Norecopa, engagement with other industry-relevant professionals including NVS, NACWOs and veterinary surgeons with expertise in marine fish.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The applicants research group remains up to date in the latest literature via research group meetings, reviewing for journals, and because the applicant is a member of the institutional AWERB. In addition to integrating advances this way, our group is also seeking to find and contribute advances to the field. The applicant is the primary supervisor for a PhD studentship investigating practice in wild fish tagging. The studentship seeks to generate new understanding regards the use of local anaesthesia,

the role of deck irrigation of fish undergoing procedures, and the histological and immunological response of fish to darting, including wound healing. Findings will be integrated into this A(SP)A Project Licence via amendments. In addition, the applicant is the primary supervisor for a PhD studentship that seeks to use computational flow dynamic modelling to understand the effects of drag generated by external tags. New understanding from that research will help to optimise the positioning of external tags on animals. As such, the applicant is at the forefront of seeking welfare related improvements in wild teleost and elasmobranch science, with active projects to refine and share information to improve practices across the sector.

109. Modelling and Therapeutics for Autoimmune Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Autoimmune polyglandular syndrome type 1, autoimmune disease, adeno-associated virus, Autoimmune regulator gene, Type 1 diabetes

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to transfer gene(s) to patients (a technique known as gene therapy) that present with autoimmune diseases, i.e. diseases where one's own body starts to react against itself and may result in killing cells required to live a healthy life. We will perform experiments first using a model of such diseases e.g. autoimmune polyglandular syndrome type 1 (APS1) and if successful will use our new therapy on more common autoimmune diseases e.g. type 1 diabetes and hair loss disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Gene therapy could be one of the best and safest medical approaches to treat or prevent disease by correcting the broken gene(s). Here we propose to transfer defective gene(s) in autoimmune diseases that are broken with a correct and functional copy. The first example is a rare condition called autoimmune polyglandular syndrome type 1 (APS1) caused by defect of a single gene called the autoimmune regulator (AIRE) gene. Treatment of APS1 patients can be very complicated as patients can present with multiple organ specific diseases as well as infection that can lead to the death of a patient. Transferring AIRE gene into patients in early age would prevent the onset of all these symptoms. We will also use the same gene transfer technology to treat patients with type 1 diabetes. There were 382 million people suffering from Type 1 diabetes (T1D) worldwide in 2013 and this is expected to reach 592 million by 2035 (WHO Bulletin No. 312, 2016) and until now there is no cure for these patients because these therapies do not target the main cause for the disease. Here we propose to transfer T1D candidate genes into an animal with T1D then if the technique is safe, we can transfer it to T1D patients.

What outputs do you think you will see at the end of this project?

At the end of this project, we will be able to confirm the efficacy of AIRE transfer for clinical applications. We will begin to organise clinical trials in children with the help of the National Organisation for Rare Disorders (NORD) and The APS1 Foundation. Gene transfer to diabetic mice will allow us to generate valuable data in term of treatment of T1D patients. Pilot study will be organised with our clinician colleagues after undertaking regulatory toxicity studies that will be performed by a Contract Research Organisation (CRO). In term of publications, we expect to publish at least 3 publications in journals with high impact factors over the next 3-5 years.

Who or what will benefit from these outputs, and how?

The patients with APS1 will benefit from the outcome of this project as the current treatment is challenging. The current treatment for T1D patients is insulin injection or more recently stem cell transfer. These approaches are based on treatment after the onset of the disease but here we propose gene transfer to prevent the destruction of beta-cells in the pancreas before the onset of the disease. This innovative approach is poised at the interface of basic and clinical research and if successful, we will be able to move swiftly to the first studies in children with the appropriate regulatory approval. In both APS1 and T1D cases the impact of gene transfer outputs will be seen only when the projects have been completed.

How will you look to maximise the outputs of this work?

By working at the early stage of the project with:

1) The appropriate regulatory committees for the clinical development phase of the project

2) APS 1 Foundation that has a register of APS-1 patients all over the word, including in the UK, and will facilitate access to UK APS-1 patients, via UK and NORD consultants, when it comes to setting up a clinical trial.

3) Working with colleagues/clinicians within international organisations in the field of diabetes.

Species and numbers of animals expected to be used

- Mice: 740

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

APS1 NOD (non-obese diabetic) mice develop spontaneous disease similar to what is found in humans. We will be using animals prior to overt clinical symptoms that leads to death. Closely monitoring their weight loss between 6-14 week corelates with the intensity of clinical disease that can be found in their pancreas and lung tissues.

We are planning to perform intrathymic/i.v. injection at soon after birth to help educate the immune cells that arise in the thymus at this early age. The animals will be humanly killed when they are approximately 12 weeks-old before the appearance of pronounced clinical symptoms

NOD mouse model:

To measure the efficacy of our gene therapy approach we need to keep mice until they develop hyperglycaemia (raised blood glucose level, similar to early diabetes in humans) as measured by blood samples, humane endpoint would be mice presenting with blood glucose of 15mM.

Typically, what will be done to an animal used in your project?

For NOD APS1 mouse model

- Animals and age: Aire mice post-natal (one day and 1 week old)
- Treatment: 1) injection of a correct and functional copy of the AIRE gene or control mice will receive an injection of sterile solution
- Time frame of the experiment: up to 12-weeks after the injection of the gene
- Every week, a small volume of blood (10 microliters) will be taken from each animal for analysis
- On the final day of the experiment, the animals will be killed and the tissue harvested for analysis.

NOD T1D model

Animals and age: Aire mice post-natal (one day1 week and 5 weeks old)

- Treatment: 1) injection of a correct and functional copy of a gene(s) or control mice will receive an injection of sterile solution
- Time frame of the experiment: up to 30-weeks (15 weeks for controls) after the injection of the gene(s)
- Every week, a small volume of blood (10 microliters) will be taken from each animal for analysis
- On the final day of the experiment, the animals will be killed and the tissue harvested for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

We expect treated animal to show mild or no sign of the disease in both cases including NOD Aire and NOD experimental models. However, around 14 weeks NOD Aire KO can show lung problems that can lead to significant weight loss. NOD model can present with raised blood glucose levels (hyperglycaemia) in females and males as early as 12 and 15 weeks of age, respectively, immune infiltration into the pancreatic islets, insulitis, begins much earlier.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severity for NOD Aire treated animals would be mild Severity for NOD Aire untreated animals would be moderate Severity for NOD treated animals would be mild

Severity for NOD untreated animals would be moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We have carefully considered the extent to which these experiments could be replaced by in vitro studies. It is important to consider the complexity of the thymus microenvironment. It is a complex network of interaction that comprises non lymphoid cells (e.g., thymic epithelial cells or TEC), cytokines, chemokines, extracellular matrix elements (ECM), matrix metalloproteinase and other soluble proteins. It is not feasible to replicate this in in vitro set up using cell cultures.

Which non-animal alternatives did you consider for use in this project?

We used and are using cell lines including human thymic epithelial cells TEC 1A3 and mouse thymic epithelial cell line 4D6 to optimise all our techniques before working on animal

Why were they not suitable?

It is important to consider the complexity of the thymus microenvironment. It is a complex network of interaction that comprises non lymphoid cells (e.g., thymic epithelial cells or TEC), cytokines, chemokines, extracellular matrix elements (ECM), matrix metalloproteinase and other soluble proteins. It is not feasible to replicate this in in vitro set up using cell cultures.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number was estimated according to the proposed experimental protocols in our previous gene therapy work.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The numbers of animals used will be minimised. Our general approach is to test hypotheses in in vitro systems prior to more formal testing in mice. We aim first to test the efficiency of gene transfer vectors in cultured cells and having observed a positive effect, only then we will move on to in vivo approaches.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our studies of knock out mice are usually staged with the aim of obtaining key pilot data on the efficacy of our approach, and in order to perform power calculations to determine an appropriate sample size for subsequent investigations. We aim to design experiments that maximise use of animals for data collection.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically altered mice are best suited to model the aspects of APS1 disease that we wish to investigate. There are no other suitable vertebrate models that are available to us, which will be suitable for our proposed investigations. We have carefully considered the method of antigens delivery, use of patch is non-invasive method. Close monitoring will be in place for animals under our studies.

We will observe the animals for morbidity, mortality, injury and intact of food and water supported by close monitoring of body weight. Our protocols cause the least pain, suffering, distress or lasting harm consistent with achieving our scientific objectives and are limited to no more than moderate severity.

Why can’t you use animals that are less sentient?

The most obvious and less sentient model to consider was Zebrafish as the thymus is present in all gnathostome vertebrates because it is a key organ for generating adaptive

immune system that leads to generation then maturation of T-cells. In mammals, thymus development is completed at birth and thymic selection also starts during embryonic development and continue after birth. Thymus involution starts in early life. In contrast, in Zebrafish thymus growth peaks at puberty and age-related involution starts at reproduction. As our gene therapy approach is based on preventing rather than treating the disease, it would not be possible to use a model such as Zebrafish where thymus development reaches its peaks at puberty.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Close monitoring will be in place for animals under our studies. We will observe the animals for morbidity, mortality, injury and intact of food and water supported by close monitoring of body weight. Our protocols cause the least pain, suffering, distress or lasting harm consistent with achieving our scientific objectives and are limited to no more than moderate-sever side effect as described above. Animal welfare is our priority, and our establishment has well-resourced and well-equipped modern facilities animal house. Where applicable we will use barrier systems (IVC's) for maintaining health of animals. We will be using ultrasound to guide needle to site of injections in the thymus. This method is accurate and allows us to avoid repetition. It is also reproducible which minimise error margin between individual animal. We will be using intrathymic injection using ultrasound to guide the needle to the injection sites of the thymus which will reduce the pain and minimise experimentation errors.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our institution has developed standard operating procedures (SOPs) which are appropriate for mouse. All surgical procedures will for the LASA guidelines, "Guiding Principles for Preparing and Undertaking Aseptic Surgery, 2017" (https://www.lasa.co.uk/current_publications/)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

NC3Rs regional programme manager, attending 3Rs workshops, conferences, frequently searching the NC3Rs website for the latest updates

110. Immune Cell Influence on the Development of Ovarian Cancer

Project duration

2 years 0 months

Project purpose

- Basic research

Key words

ovarian cancer, macrophage, cholesterol, adipocyte, omentum

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To examine macrophage cholesterol metabolism during ovarian cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Ovarian cancer is the most deadly gynaecological cancer, which is often discovered late in patients. We need to better understand the early stages of this disease to determine how to better diagnose the disease, which would mean that available treatments could be given earlier and have a greater chance of curing the cancer. It is also clear that currently available treatments for ovarian cancer could be better and more specific, and we can only develop new treatments that work better if we first understand how the cancer develops

and why the immune system is unable to kill it. We want to understand how immune cells called macrophages promote the spread of ovarian cancer to the omentum (the main site of metastatic tumours) and prevent clearance of cancer cells. Macrophages are the most numerous immune cells in the tumour and omentum, and when they are depleted, cancer is better controlled and spread reduced. Yet, the pro-tumour functions of macrophages remain incompletely understood. Our preliminary data implicates cholesterol metabolism as a mechanism that controls macrophage function in the context of cancer. This project will test how this works, focusing on the early stages of cancer development where cancer cells first invade the omentum.

What outputs do you think you will see at the end of this project?

This work will generate new insights into how immune cells called macrophages influence the spread and severity of ovarian cancer. Previous work investigating why ovarian cancer spreads has shown that macrophages are one of the culprits, but exactly how they do this isn't known. Our research will provide answers by carefully examining macrophage behaviour during ovarian cancer. We will share these new findings in publications and presentations at conferences, seminars and science festivals. The research outlined in this licence will also generate preliminary data that will act as a foundation for our future work in grant applications that aim to translate our research efforts into the clinic.

Who or what will benefit from these outputs, and how?

In the immediate to short-term, the main benefits of this research are for academics and clinical scientists, as the project will provide new insights into how macrophages function during ovarian cancer, leading to new clues as how to best approach treatment strategies for this disease. This work will help build our existing collaborations with clinicians and oncologists, leading to the development of a translational research program that utilises the information we gain from our mouse models to benefit cancer care for humans in the long-term.

How will you look to maximise the outputs of this work?

We currently collaborate with clinical scientists to ensure our research is clinically-relevant and has the best chance of generating results that will be useful for patients in the long-term. Our collaborative nature mean that our data reaches a wider audience, as we will regularly present our findings to our own department(s) as well as our collaborators. Regardless of the outcomes of this work, all data will be published in journals and presented at conferences. This is because our planned approach aims to refine the methods used to study early engagement of immune cells with cancer, which will be beneficial for other researchers and applicable to a variety of biological questions. Hence, whether we find a specific role for macrophage cholesterol metabolism or not, our results will still be useful to others and worthwhile reporting as new findings or as a published protocol.

Species and numbers of animals expected to be used

- Mice: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We want to use mice for these studies because:

1) Mice can be genetically-altered which means we can manipulate the expression or function of the genes we are interested in. Our analysis of genetic data from ovarian cancer patients indicates that macrophages (an immune cell) in the tumour highly express genes involved in cholesterol metabolism, but it isn't clear what this means in terms of the development or severity of the cancer. The only way we can determine whether cholesterol metabolism in macrophage is important is to change the genes controlling this process. This is only possible with mice. Our initial experiments (outlined in this licence) will aim to examine cholesterol metabolism in macrophages during ovarian cancer so that we can pin- point the specific genes and pathways that may be involved with processes such as metastasis and cancer cell invasion of the omentum, and would be most relevant to manipulate in future studies with transgenic mice.

2) We can study the very early stages of ovarian cancer in mice. One of our hypotheses is that macrophages in the omentum (the place that cancer cells first spread to from the tumour) are important for helping cancer cells grow and develop into tumours. Patients are often diagnosed after this process has already started, so we need to use a mouse model to look at the processes involved when cancer cells first start entering the omentum.

All of the above require adult female mice because we need mature ovaries to initiate the ovarian cancer model.

Typically, what will be done to an animal used in your project?

Mice would typically be injected with an ovarian cancer cell line (into the ovary) and then monitored for the development of tumours and evidence of tumour-associated side-effects (e.g. signs of abdominal fluid build-up). Monitoring will involve regular weighing and palpitating abdominal examinations. In addition, we plan to pilot a model that would allow us to monitor tumour development using a non- invasive in vivo imaging system, where tumour cells produce luminescent signals that can be detected using a scanner. Mice would be anaesthetised in this case to be imaged under the scanner, allowing for more consistent measurement of tumour development and imaging of early tumour invasion. This approach will enable us to take mice at an earlier time point (when tumour cells first

start to spread) that we may not otherwise be able to detect (i.e. before tumour development) and reduce our variability by only examining mice that are at the right stage of tumour progression for our studies. Thus, this approach represents both a refinement and reduction of animal use. At various time-points after injection of cancer cells, we will humanely cull the mice and isolate macrophages from various body sites (e.g. omentum, ovary) and analyse them for expression of cholesterol metabolism genes, lipid content and/or interaction with cancer cells.

What are the expected impacts and/or adverse effects for the animals during your project?

The injected tumour cells can grow rapidly in mice which we will monitor carefully using non-invasive imaging and clinical scoring techniques. Mice experience changes to body weight and develop abdominal tumours, we will monitor carefully for these outcomes and particularly be watchful for evidence of developing abdominal fluid (e.g. abdominal swelling, sudden weight change) at which point animals will be humanely euthanized using a schedule 1 method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

As our mice will undergo a surgical-based technique to inject the ovarian cancer cells, and we expect that 100% of injected mice will go on to develop tumours, our mice will experience a moderate severity due to the cumulative effect of the procedures and tumour growth. At each stage, we will use anaesthesia and pain relief where necessary (e.g. before and after surgery) and monitoring techniques (e.g. imaging for tumour growth) to limit severity to moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are several challenges in our proposed research that cannot be solved using patient samples or in vitro models, and only mice are suitable. Those challenges are:

1) Understanding how macrophage cholesterol metabolism is regulated in different body sites and how ovarian cancer influences this. Macrophages are very sensitive to their

environment, therefore the signals they receive in the omentum are different to the signals they receive in the ovary, and this changes how the macrophages function. The omentum is very fatty, hence the macrophages must be able to deal with the excess cholesterol they are exposed to. We think this affects how these macrophages behave when confronted with ovarian cancer cells, and prevents them from activating properly. To test that theory, we need to be able to compare macrophages from fatty sites like the omentum to sites where there is little to no fat (e.g. ovary, spleen), in mice with ovarian cancer and healthy controls. This is only possible in a whole body system, such as the mouse.

2) Determining whether and how macrophages influence the spread of ovarian cancer. We think that targeting macrophages might provide a new strategy to preventing ovarian cancer cells from getting into the omentum and developing tumours. The only way we can test out this new strategy is to first understand how macrophages are involved with the spread of cancer. It is not yet possible to do this using an in vitro system, therefore mice are essential for helping us to model the spread of ovarian cancer cells so that we can examine the role of macrophages in this process.

Which non-animal alternatives did you consider for use in this project?

We have considered and use several non-animal alternatives for this research:

1) Publically-available datasets. We access and analyse large datasets of gene expression data from ovarian cancer patients to determine which genes are most highly expressed by macrophages in tumours. We have used this approach to develop our hypotheses that cholesterol metabolism in tumour-associated macrophages might be important for cancer development.

2) Macrophages and omentum samples from patients. We have begun experiments that use tissue samples taken from patients during standard care surgical procedures. From these, we hope to culture macrophages and study their function using cell culture methods.

3) Tissue culture models. We currently run experiments where we co-culture macrophages (either from a cell line or made from blood samples taken from healthy humans) with cancer cell lines and/or human ascites fluid. In these experiments, we test how manipulating macrophage metabolism affects their interaction with cancer cells and their responses to signals within human ascites fluid.

Why were they not suitable?

All of the non-animal alternatives have been useful for aspects of our research but they crucially fail to provide answers about the early stages of ovarian cancer in the omentum. In patients, we can only access samples and data after tumours have established because this is when patients receive surgery (to remove tumours), and patients have often already received chemotherapy which significantly alter and/or deplete the macrophages we want

to study. With our tissue culture and organoid systems, we can't analyse how cancer cells spread between body sites (i.e. metastasis) or how macrophages are involved with that. Macrophages change their function depending on the signals

they receive in tissues, and this is usually very different between organs. Therefore, macrophages in the ovary are distinct from macrophages in the omentum or tumour. The only way we can determine whether these differences are important or relevant is to directly analyse macrophages in these tissues, which we can't yet model with a tissue culture system.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated numbers based on running several pilot studies (around 10 pilots with 5-10 mice per pilot) to analyse variability in the parameters we intend to measure. Some of these parameters we have measured in other systems so have a good idea of the number of mice needed to generate statistically robust results (around 3-6 mice per group). Based on this and the fact that we will replicate all experiments at least twice for reproducibility, we have estimated enough mice to cover all of these types of experiments at the larger group size.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will collect multiple read-outs from each mouse used in our studies, and then use the data to make connections between the different read-outs. For example, we will analyse macrophage cholesterol content from a number of different tissue sites (e.g. ovary, omentum, peritoneum) which will help us determine how different organs change macrophage cholesterol metabolism (a central aim for this research). In some experiments, we will also bank isolated tissues (either as stored genetic material or frozen tissue blocks) that we can analyse at a later date once our research becomes more advanced, without having to use new mice.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Genetically-altered lines are bred so as to provide wild-type littermate controls alongside the test mice, which will ensure we do not breed more lines or mice than required. These

lines are also used to generate animals for other projects so we are not duplicating breeding efforts for this project.

Pilot studies are the primary way in which we will determine mouse group sizes in future experiments. We will adjust the number of mice used per experiment (if required) should the pilot study data determine that this is needed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will inject cancer cells into mice using the intrabursal route (injection directly into ovary). This ensures primary tumours develop in the ovary, which more closely mimics human disease and is associated with less adverse effects for the mouse. Cancer cells could also be injected by other routes (e.g. subcutaneous, intraperitoneal), but these methods are often associated with more severe side effects such as rapid build up of abdominal fluid causing distension and discomfort.

We will also use luciferase-expressing cancer cells for some experiments, which will allow us to monitor the size and spread of tumours in anaesthetised mice using a scanner. This method has been shown to detect tumour growth as early as 1 week post-transplant, meaning we can study early cancer spread and monitor the development of cancer in mice over time, which will reduce the number of mice needed for experiments by increasing our statistical power in kinetic studies (e.g. examining tumour spread in same animals over multiple time points) and limiting the need to use higher numbers of mice for terminal end-point studies. This technique will also identify any animals with large tumours that may not be visible/evident by standard monitoring techniques (such as changes to body weight), enabling the possibility of taking animals earlier when most scientifically useful.

Why can’t you use animals that are less sentient?

We want to monitor cancer cell spread around the body and how macrophages are involved with that. This is an active process that occurs over days/weeks, which is why we need to use live animals.

Macrophages come in many different types as they become specialised to different organs during embryonic development. Macrophages in fish or insects do not have the same level

of specialisation as mammals, and this macrophage specialisation is important for how diseases develop. For that reason, we must use adult mice so that we can study these specialised macrophages and their role in cancer.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Part of our aim in this project is to develop a refined method of monitoring tumour growth and spread using a luciferase-expressing cancer cell line and imaging. This will reduce the number of mice needed to gain results, but it also presents an opportunity to refine the model as it may help us identify mice with rapid tumour growth prior to the development of clinical or outward symptoms (e.g. early stages of development of abdominal fluid). We will also correlate our imaging results with other monitoring measures (e.g. weight gain) to help us develop a set of monitoring variables that accurately predict the development of the cancer in mice, ensuring we do not exceed severity greater than is necessary for our scientific questions.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are several recent publications (listed below) outlining different models of ovarian cancer in mice, including studies that have directly compared engraftment rates and median survival times for different cancer cell lines (e.g. ID8 vs ID8-luciferase). From this data, we have selected the ID8 intrabursal injection model as this has been shown to have greater physiological relevance, slower build-up of abdominal fluid and more accurate modelling of human disease. We will also refer to ARRIVE 2.0, LASA, NC3Rs and PREPARE guidelines in the design of our experiments.

References:

- Zakarya, Razia et al. “Modelling Epithelial Ovarian Cancer in Mice: Classical and Emerging Approaches.” International journal of molecular sciences vol. 21,13 4806. 7 Jul. 2020, doi:10.3390/ijms21134806

- Hernandez, Lidia et al. “Characterization of ovarian cancer cell lines as in vivo models for preclinical studies.” Gynecologic oncology vol. 142,2 (2016): 332-40. doi:10.1016/j.ygyno.2016.05.028

- Baert, Thaïs et al. “The dark side of ID8-Luc2: pitfalls for luciferase tagged murine models for ovarian cancer.” Journal for immunotherapy of cancer vol. 3 57. 15 Dec. 2015, doi:10.1186/s40425-015-0102-0

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I regularly receive 3Rs updates from local mailing lists and email alerts which disseminate information about new models, protocols and group meetings. I also set up 'google alerts'

for new research of interest that is then directly emailed to me. I have recently set up such an alert for 'macrophages in ovarian cancer' and 'ovarian cancer mouse models' to help me keep up to date with new research and model refinements as they are first announced. I am also working closely with collaborators who have developed non-animal alternatives (e.g. organoids) that may be relevant for aspects of my research. I intend to work closely with them to develop these models in a way that is useful for our work to further reduce our animal usage.

111. Next Generation Lipid Nanoparticles for Future Gene Therapies

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

lipid nanoparticle, gene therapy, tissue specificity, cell specificity

Animal types	Life stages
Mice	adult, juvenile, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop lipid nanoparticle (LNP) platform technologies capable of delivering nucleic acids (NA) to 3-5 distinct target tissues and/or cells in the body. Initial target tissues/cells will include hepatocytes (liver), liver sinusoidal endothelial cells (liver), T cells (spleen/bone marrow) and dendritic cells (muscle). LNPs arising from this project will expand and enable the development of novel gene therapies against significant human diseases. Target diseases will include infectious diseases (vaccines), cancer (immunotherapy), protein deficiencies (protein replacement) and autoimmune diseases (immunotherapy).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Gene therapy aims to modify or manipulate gene expression within living cells to treat or cure disease. This can involve 1) replacing a disease-causing gene with a healthy copy of the gene (gene editing); 2) inactivating a disease-causing gene that is not functioning properly (gene silencing); 3) introducing a new or modified gene into the body to help treat a disease (gene expression); or 4) a combination of the above. To achieve this in patients, delivery systems are required to protect, transport and deliver highly charged, immunogenic and membrane impermeable NA therapies to target cells and tissues in the body.

Lipid nanoparticles (LNPs) - self-assembled spheres of fats, 1/1000th the diameter of a human hair - can entrap, protect and deliver nucleic acids (NA) in patients. Marketed LNP-based gene therapy products, including the mRNA prophylactic vaccines against Covid-19, have emphatically shown these gene delivery systems are safe and effective, can be developed at unprecedented speed, manufactured at scale and produced at low cost. The question now is how quickly can LNP-based gene therapies be developed to treat other significant human diseases.

The outstanding challenge is delivery. Current LNP technologies are optimised to deliver NA to the liver (hepatocytes), following systemic administration, and lack the tissue/cell precision and potency required for effective gene therapy of most diseases. To overcome this delivery barrier, a combination of 1) rational LNP design, based on pre-existing knowledge, 2) improved in vivo screening methodologies and 3) comprehensive understanding of critical LNP interactions within the body, is required. Collectively, the objectives of this project meet these three criteria so as to expedite the discovery of potent and precise LNP-NA technologies that will form the basis of future gene therapies against significant human diseases, including infectious diseases, cancer, protein deficiencies and autoimmune diseases.

What outputs do you think you will see at the end of this project?

By the end of this 5 year Project, three key outputs are expected.

1) New LNP platform technologies individually demonstrating safe and effective NA delivery to 3-5 distinct tissues and/or cells of the body. These delivery platforms will be leveraged through academic and commercial partnerships for further development of gene therapy products against a wide range of human diseases (e.g. vaccines for infectious diseases, cancer immunotherapies, inherited genetic diseases).

2) New method(s) to reduce the number of animals required to screen LNP-NA formulations in vivo. Validated methodologies will be published in high impact academic journals and presented at (international) conferences.

3) Improved understanding of the biological mechanism(s) that underpin LNP safety, performance and fate within the body. This information will be published in high impact academic journals and presented at (international) conferences.

Who or what will benefit from these outputs, and how?

LNPs arising from this project will expand the repertoire of tissues and cells into which therapeutic genes can be safely and effectively delivered.

In the short term, these technologies will be used to develop (outside of this Project Licence) novel gene therapy products against significant human diseases, including vaccines for infectious diseases, cancer immunotherapies and protein replacement therapies. This will be done through non-exclusive academic and commercial partnerships. Partners will benefit from gene delivery technologies that enable them to validate their own NA therapy against a specific disease target. We will benefit from partner expertise of a specific disease and/or NA therapeutic modality that will enable us to further optimise LNP designs.

Longer term, it is expected that LNP platform technologies developed during this project will form the basis of future gene medicines that will be used to treat many significant human diseases.

Beyond the development of new tissue/cell specific gene delivery technologies, new methods to reduce animal numbers needed to screen new LNP-NA formulations, and new knowledge to explain the fate of LNP-NA delivery systems in vivo, will improve and streamline research outcomes throughout the nanomedicine/gene therapy research community. This will expedite the development of new and effective nanomedicine/gene therapy products.

How will you look to maximise the outputs of this work?

As 'plug and play' gene delivery technologies, each LNP design can be re-purposed to deliver different NA therapies to the same target tissue or cell type. Therefore, it is expected that multiple partnerships, each focused on a specific disease and/or NA modality, will be established for each individual LNP platform technology developed in this project. Overall, we expect >10 LNP-NA gene therapy products, including new vaccines for infectious diseases, cancer immunotherapies and protein replacement therapies, to be in clinical evaluation by the end of this 5 year project. To enable this model, options to licence LNPs will provide a viable route to market but will not restrict the re-use of a specific LNP technology for alternative disease targets (or for the same disease target but alternative therapeutic NA).

All key results, new methodologies and new knowledge will be disseminated though patent applications, conference talks and/or high impact journal publications to maximise knowledge output from this Project. Where appropriate, publications will report unsuccessful as well as successful results.

Species and numbers of animals expected to be used

Mice: 8700 (as follows WT = 7200; GA = 1500)

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are well established animal models to evaluate pre-clinical pharmacological, safety and toxicity profiles of a broad range of drug substances, including LNP-NA technologies. Standard protocols for assessing reporter LNP-NA biodistribution and transfection potency in mice are defined and LNP-NA doses required to produce a high (i.e. easily detectable) level of reporter gene expression do not adversely affect the animals.

Outbred mice will be used for preliminary pharmacological screens to ensure results are reliable across a genetically diverse population. Inbred strains, with reduced genetic variance and consistent cell surface receptor expression, will be used to confirm results in the minimum number of animals. GA (conditional) reporter lines, many only available in mice, will be used to screen specific NA therapies, such as small interfering (si)RNA knockdown of reporter genes, as well as to validate new screening methodologies aimed at reducing the number of animals required for LNP-NA in vivo screening. GA mice - lacking one (or more) specific receptor(s)/protein(s) - will be used to confirm biological mechanism(s) underpinning LNP target tissue/cell tropisms. In this project, it is expected that relevant genes will be associated with endogenous lipid transport, metabolism and clearance pathways. GA animals lacking specific lipid transport and metabolism receptors/serum proteins are viable and fertile and do not show an adverse phenotype. Many of these knock out models have only been generated in mice.

Adult mice will be used throughout this project to avoid false positives arising from specific developmental phenotypes.

Typically, what will be done to an animal used in your project?

New LNP designs will be assessed following a single injection of a reporter LNP-NA (e.g. LNP encapsulated mRNA encoding firefly luciferase) formulation (100% of mice). As a result, targeted cells within the animal will ‘glow’ and can be imaged and quantified over time.

Non-invasive (bioluminescence/fluorescence) imaging (80% of mice) will be performed up to a maximum time point of 168 h (one week) post-injection. During imaging (typically 5-15 mins, maximally 30 mins), animals will be under general anaesthetic (gaseous). Imaging will be performed a maximum of three times over the course of an experiment with no less than 6h between individual imaging timepoints. In the case of bioluminescent reporter proteins (eg. firefly luciferase), required substrates (e.g. luciferin) will be injected shortly before imaging.

Blood samples will be taken from the tail vein up to a maximum of 15% total blood volume. Local anaesthetic will be applied to the area twenty minutes before the bleed and a maximum of three withdrawals will be taken from any one animal over the course of an experiment. Blood samples will be used to assess LNP-NA pharmacokinetics and safety (e.g. clinical chemistry, haematology, serum cytokine levels).

At the **end of the experiment**, animals will be killed by terminal anaesthesia. Terminal blood samples will be collected, and organs may be perfused, for downstream analysis (eg. histology, FACS analysis).

No surgical procedures will be performed on this Project Licence.

What are the expected impacts and/or adverse effects for the animals during your project?

Screening assays will be performed following published protocols. Typical LNP-NA doses (0.1-1 mg/kg reporter RNA) produce high levels of reporter gene expression without producing an adverse effect in animals. All experiments will be benchmarked using control LNP-NA formulations (e.g. OnpattroTM).

These formulations have well documented dose-dependent safety profiles in mice and are safe for use in humans. In general, it is not expected that animals will experience any adverse effects from LNP-NA administration beyond the transient pain of the injection itself and blood sampling procedures.

However, in developing LNP-NA formulations with specific target tissue/cell tropisms, LNP compositions will be screened that contain compounds not been previously tested in animals. While these compounds will generally be synthetic iterations of chemicals previously shown to be safe in animals, we cannot rule out the possibility of new LNP designs causing an unexpected adverse effect in animals. To minimise the numbers of animals exposed to this risk, pilot in vivo experiments (2 animals) will be performed for any LNP-NA containing compound(s) not previous tested in animals.

Following sample administration, these animals will be closely monitored for clinical signs. In the event of an adverse reaction, the experiment will be immediately terminated and the LNP/compound in question ruled out of any further studies.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Breeding and maintenance of GA animals (approx. 15% of all animals used in this project) - Approx. 95% expected to be below threshold. Approx. 5% may experience mild severity if two biopsies are required for identification.

LNP-NA administration - **mild** (100% animals)

Non-invasive imaging - **mild** (80% animals) Blood sampling (tail vein) - mild (80% animals)

While the severity of each individual procedure during the course of an experiment is considered mild, the cumulative procedures and repeated animal handling over a relatively short period of time (typically 48-72 h), means the overall expected severity should be considered moderate. Clinical signs will be closely monitored and if experiments regularly reach moderate severity, procedures will be adapted to reduce animal suffering and harm. For example, the number of imaging timepoints and/or blood samples will be reduced.

What will happen to animals at the end of this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

LNP gene delivery technologies developed in this project are intended to be further developed (beyond this Project Licence) into gene therapy products to treat human diseases. It is crucial, therefore, to screen and optimise reporter LNP-NA delivery systems in models that accurately discriminate the systemic physiology, immunology and cellular complexity of the human body. Determinant factors such as blood flow, functional innate and adaptive immune system, diverse blood components and the complex arrangement of blood vessels, lymphatics and various tissue architectures, have all been shown critical in determining the fate and safety of an LNP-NA in vivo. System simplification of any of these determinant factors runs the significant risk of obtaining false positive or negative results. The challenge is therefore how to balance the (high throughput and desirable) screening of new LNP-NA formulations in simplified, non-animal alternatives so as to inform, but not mislead, in vivo selection criteria.

Which non-animal alternatives did you consider for use in this project?

1. In silico modelling to enable de novo optimisation of LNP-NA chemical composition and structure so as to predictably enhance potency and targeting precision in vivo.

2. In vitro studies using cultured cells to assess LNP-NA targeting, transfection potency and safety.

3. More complex, organ-on-a-chip (OOAC) and stem cell derived, three dimensional (i.e. organoid) in vitro systems to assess LNP-NA targeting, transfection potency and safety.

Why were they not suitable?

In silico optimisation

Given the (large) size and complexity of a self-assembled LNP-NA (consisting of >100,000 individual molecules), current molecular dynamic simulations are not yet capable of de novo modelling LNP structures at the required (bio)molecular detail to accurately predict LNP-NA in vivo fate. Using acquired in vivo datasets, however, in silico machine learning will be employed in this Project to inform on specific lipid chemistries. As an example of this, we are currently able to predict, with good accuracy, specific lipid structures that will preferentially direct an LNP-NA either to the liver or to the spleen. As this Project progresses and more in vivo data is acquired, the predictive power of machine learning algorithms will improve, and with it, the ability to rationally design, from the bottom up, LNP-NA with enhanced tissue/cellular precision and/or potency in vivo.

In vitro assays using cultured cells

As static experiments against a single cell type, in vitro screens using cultured cells are far removed from modelling the complex and dynamic in vivo interactions that determine LNP-NA in vivo fate.

However, through careful choice of cell type, dose-dependent cytotoxicity screens - benchmarked against 'gold standard' and safe LNP-NA formulations - can provide valuable information on potential LNP in vivo safety and appropriate dosing in animals. In this Project, dose-dependent cytotoxicity assays will be performed for all LNP-NAs containing compounds not previously tested in animals. Cultured cells will be selected based on known/expected LNP interactions in vivo, e.g. with innate immune cells (e.g. macrophages (THP-1)) and/or target cells (e.g. hepatocytes (Hep2G)). This approach aligns with guidance from the US Nanotechnology Characterisation Laboratory (NCL) that assists regulatory agencies in the approval process of medical applications containing nanomaterials.

The use of more complex, organ-on-a-chip (OOAC) or organoid in vitro models

To overcome the shortcomings of simple cell culture assays, significant efforts are being made to create sophisticated in vitro systems, such as organoids or OOACs, to more accurately reflect the microenvironment of individual organs. However, as isolated organ-like systems, organoids/OOACs cannot model determinant and dynamic LNP-NA interactions within other organs, with immune cells and/or with systemic blood components. Furthermore, at an organ level, the physiological complexity of current organoid/OOAC technologies is often insufficient to inform on key LNP-NA interactions.

Taking the liver – the major organ within which systemically administered LNP-NAs accumulate – as prescient example, no liver organoid/OOAC has yet shown 1) correct macro- (e.g. hepatic lobules) or micro- (fenestrated sinusoids) liver tissue architectures; 2) co-culture of all five major liver cell types; 3) incorporation of both red and white blood cells (save for Kupffer cells), and/or 4) realistic liver vascularisation. However, all these features are critical in determining the intrahepatic fate of systemically administered LNP-NAs.

For these reasons, current organoid/OOAC technologies do not further inform on the efficacy and safety of systemically administered LNP-NAs, above and beyond what can be gained from simpler cell culture assays. These more complex systems will not therefore be incorporated as part of an in vitro triage prior to animal experiments. That said, organoid/OOAC technologies are constantly improving and, in particular, efforts to connect individual organoids (to enable more complex modelling of the systemic in vivo environment) are beginning to emerge. Through our network of academic and industrial partners, and through alerts and newsletters from organisations such as NC3Rs (https://www.nc3rs.org.uk), Alternatives to Animal Testing (https://caat.jhsph.edu) and Norecopa (https://norecopa.no), as well as updated NCL guidelines, I will ensure to keep fully informed and up-to- date with the latest developments regarding these in vitro technologies. Any arising technology that enables us to gain more accurate information on the fate and/or safety of an LNP prior to animal experiments will be incorporated within our discovery pipeline.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The majority of experiments performed in this Project will be exploratory screens to identify novel LNP- NA formulations with enhanced cell/tissue precision and/or potency in vivo. For these comparative studies, the aim is not to overcome a specific (reporter gene expression) threshold value but rather compare LNP-NA targeting and transfection potencies between different experimental groups, and against benchmark controls. Statistical analysis is therefore not a prerequisite and group sizes (n=4) used in reporter LNP-NA screens in mice are typically smaller than those used in the therapeutic assessment of e.g. a new drug entity. In addition, variability within experimental groups is expected to be low as all LNP-NA formulations must meet strict biophysical quality control criteria to ensure sample homogeneity.

A typical LNP-NA in vivo screen will involve: [(13 experimental LNP-NA formulations x 1 dose) + (1 benchmark LNP control x 1 dose) + (1 PBS control x 1 dose)] x n of 4 = 60 wild-

type mice. We anticipate undertaking approximately 24 such experiments per year. Total animals used for comparative LNP-NA screens over the course of this project will be approximately 7200.

In addition, GA mice (approx. 1000) will be used to validate new screening methodologies and to confirm biological mechanisms underpinning LNP-NA fate. For the latter, statistical analysis between experimental groups will be required. In these cases, existing data, as well as data generated from preliminary screens, will inform on minimum numbers required to confirm statistical significance. From experience, relatively small group sizes (n=4-6) generally provide enough statistical power to show significant differences between wild-type and GA mice lacking a specific receptor/protein required for LNP-NA cell/tissue targeting.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Given the complexity and potential chemical diversity of a self-assembled LNP-NA, informed design approaches should be employed to avoid inefficient empirical screening of LNP-NA formulations in vivo. To this end, I will ensure 1) general nanoparticle design guidelines are followed, 2) strict biophysical quality control criteria are met, and 3) a clear and demonstrable hypothesis, based on pre- exisiting knowledge and/or data generated in vitro and/or in silico, supports screening of any new LNP- NA design in vivo.

General nanoparticle design guidelines:

1. Any LNP-NA above a size of approx. 150 nm will not be progressed. Nanoparticle sizes greater than 150 nm are known to be rapidly recognised and cleared by scavenging cells of the reticuloendothelial system (primarily within the liver). An exception may be made if these specific cells (eg. Kupffer cells) are the intended target.

2. Any LNP-NA with a measured cationic surface charge of > +15 mV will not be progressed. Cationic nanoparticles are known to be toxic as a result of non-specific cellular uptake.

Biophysical quality control criteria:

1. All LNP-NA formulations must meet physicochemical criteria of a) a number-weight Z-average size

<150 nm, b) polydispersity < 0.2, and c) NA entrapment > 80%. These criteria will ensure LNP-NA formulations perform consistently (i.e. low sample variability) in vivo and are suitable for further clinical development.

As demonstrable hypotheses:

Where appropriate, a combination of in silico and in vitro optimisation will be performed. Methods will include:

1. In silico design of new lipid reagents to efficiently entrap NA payloads and direct LNP-NA systems away from the liver. Within our organisation, machine learning is currently used to rationally design lipid reagents that are 1) biodegradable, 2) efficiently entrap NA and 3) predictably modulate the surface properties of an LNP so as to redirect NA delivery to organs beyond the liver. These machine learning algorithms are informed by existing LNP-NA in vivo data sets and will continually improve as new, generated in vivo data is additionally fed into them.

2. In vitro characterisation of the adsorbed protein corona of an LNP-NA formulation through quantitative mass spectrometry analysis. This approach will be used to refine LNP compositions to bind specific serum proteins so as to alter tissue/cell tropisms. In particular, we will aim to exploit the preferential binding of serum apolipoproteins to exploit endogenous mechanisms of lipoprotein transport and metabolism.

3. In vitro assessment of cellular uptake mechanisms and endosomal escape. High resolution fluorescence microscopy experiments will be used to inform optimal LNP-NA designs regarding intracellular delivery of a NA payload. In addition, high throughput fusion assays (using model membrane systems) will be used to optimise LNP-NA endosomal membrane fusion and cytosolic NA delivery.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All breeding for experimental cohorts will be performed according to precise breeding calculations, the formulae of which are refined and updated periodically with reference to current breeding figures.

Numbers of mice used for GA breeding are frequently reviewed and altered breeding schemes are trialed to maximise productivity and minimise overbreeding. All crosses use dynamic mating systems, where the male is removed prior to littering thereby avoiding second litters and the risk of overbreeding.

For in vivo screens, the use of non-natural reporter proteins, with no/minimal endogenous background signal, minimises/eliminates the chance of false positives and reduces the number of animals required to confirm a positive result. Furthermore, by employing a combinatorial approach that directly compares multiple LNP-NA formulations in a single study, experimental variability is reduced, as are the number of positive and negative control groups required in each experiment.

Where compounds to be tested are first in animals, pilot studies will be preformed (1-2 animals) to ensure possible adverse affects are identified in the minimum number of animals.

SOPs have been written for all protocols to be performed under this project licence and will be used throughout to reduce experimental variability.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animal models used in this project

Wild-type mice

As genetically diverse animals, the use of outbred mice in preliminary LNP-NA screens will minimise the risk of false positives arising from strain specific genotypes and will ensure results are reliable.

Inbred strains, with reduced genetic variance and consistent cell surface receptor expression will be used to confirm organ/cellular distribution of LNP-NA delivery systems using the fewest possible animals. Wild-type stocks and strains have no adverse phenotype.

GA mice

Reporter lines: Transgenic reporter mice stably express a reporter protein (bioluminescent/fluorescent) in virtually all cells. These mice will be used to assess functional delivery of specific NA payloads so as to expand the demonstrated utility of developed LNP-NA delivery systems. For example, tissue specific, small interfering (si)RNA-mediated knockdown of transgenic luciferase expression.

Transgenes to be used in this project are not detrimental to the animal. Established reporter lines will be imported from other sources and selected based on having no overt adverse effects.

Conditional reporter lines: Transgenic conditional reporter mice carry conditional reporter genes (e.g. tdTomato, luciferase) that can be activated by introduction of a specific recombinase enzyme (eg. Cre). This enzyme can be mRNA encoded and delivered by an LNP. These mice may be used to validate screening methodologies that enable many LNP-NA formulations to be screened in a single animal.

Transgenes to be used in this project are not detrimental to the animal. Conditional reporter mice will be imported from other sources and selected based on having no overt adverse effects.

Knockout mice: Mice strains where a specific gene has been removed will be used to confirm the biological mechanism underpinning tissue/cell tropisms of specific LNP-NA delivery systems. For this project, it is expected that relevant genes will be associated with endogenous lipid transport, metabolism and clearance pathways. GA animals lacking specific receptors/serum proteins associated with endogenous lipid transport will be imported from other sources and selected based on having no overt adverse effects.

Methods used during the project:

For all methods used in this project, we will use the most appropriate route for the biological aims using best practice and minimum accurate dosing.

Ear clipping: GA mice need to be ear clipped for identification and the same piece of tissue is used for genotyping. Genotyping protocols have been optimised to use very small tissue samples for all types of genotyping. Ear clipping is more refined than other methods such as a tail biopsy. This procedure is expected to cause no more than transient pain to the animal.

LNP-NA administration: LNP-NA formulations will be administered by appropriate route. Local anaesthetic will be applied to the area twenty minutes before the injection to ensure minimal pain and suffering to the animal. This procedure is expected to cause no more than transient pain to the animal.

Non-invasive imaging : For non-invasive imaging of reporter proteins/probes, such as expression of a non-natural bioluminescent protein, mice will be placed inside the imaging equipment (Biospace Lab PhotonIMAGER Optima) under sustained gaseous anaesthesia. Imaging will typically last 5-15 minutes (up to a maximum of 30 mins). Animals are not expected to suffer pain, suffering, distress or lasting harm as a result of non-invasive imaging. In the case of bioluminescent reporter proteins, appropriate substrates will be administered shortly prior to imaging. This is expected to cause no more than transient pain to the animal.

Blood sampling: Blood samples will be collected, at various timepoints, up to a maximum of 15% total blood volume of the animal. Removal of this volume of blood has been shown to have no adverse effects in previous studies. Samples will be taken from the tail vein using a very small cut and mice will have local anaesthetic applied to the area twenty minutes before the bleed takes place to ensure minimal pain and suffering to the animal. This procedure is expected to cause no more than transient pain to the animal.

Cage based monitoring: The activity of mice may be recorded by non-invasive recording in their home cages (i.e. usual caging, not singly housed). This may necessitate the

insertion of a radio frequency identification chip. Subcutaneous administration of the microchip is expected to cause no more than transient pain to the animal.

Animal killing by terminal anaesthesia and terminal blood collection: Animals will be killed by terminal anaesthesia. During terminal anaesthesia, a terminal blood sample may be taken or exsanguinations performed, and mice may be perfused to enable downstream analysis of LNP-NA uptake within specific organs and cells. Following terminal anaesthesia, tissue samples may be removed with or without the administration of substances necessary for tissue preparation.

Why can’t you use animals that are less sentient?

Less sentient species, such as zebrafish (70% genetic homology to humans) have significant physiological (e.g. cold blooded, lack of serum albumin, reticuloendothelial system outside of the liver) and anatomical (eg. lack of heart septation, cancellous bone, limbs and lungs) differences to mammals. As a translational animal models to assess LNP-NA in vivo performance, these less sentient animals do not therefore accurately replicate the biological environment and in vivo interactions that will determine LNP-NA fate in humans.

Screening LNP-NAs in animals at a more immature life stage runs the significant risk of false positive results arising from specific developmental phenotypes. For example, liver targeted LNP-NA systems administered in embryonic zebrafish do not accumulate or transfect hepatocytes within the developing liver despite this fate being well characterised in higher order, adult mammals.

Experimental timeframes (to maximal reporter gene expression) are expected to be between 6 and 72 h post LNP-NA administration. This prolonged timeframe means it is not possible to perform experiments using terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For LNP-NA systems containing compounds previously shown safe in animals, animals will be monitored closely over the first 30 minutes, then once over the next 8 hours, then daily until endpoint (up to a max. 168 h).

For LNP-NA systems containing compounds not previously tested in animals, animals will be monitored continuously for the first hour after LNP-NA administration, closely over the next 4 hours then daily until experiment end. Additionally, home cage monitoring systems and video recording may be used in these cases to enable continuous monitoring of animals. If any adverse effects are observed, the experiment will be terminated immediately.

For LNP-NA administration: pain from injections will be reduced by the use of local anaesthesia.

For in vivo imaging: distress and suffering will be minimised as animals will be under gaseous anaesthesia throughout the imaging procedure.

For blood sampling: pain from tail bleeds will be reduced by the use of local anaesthesia. This licence involves no surgery.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Routes and volumes for administration of substances are taken from LASA guidelines.

The Licensed Establishment where all animal experiments will be performed has full AAALAC and ISO (9001:2015) accreditation. To conform to these standards, the Establishment must ensure a high level of quality control on all fronts including husbandry, phenotyping and administrative processes.

Standard operation procedures for all tests will be generated using data and expertise from multiple animal houses.

ARRIVE guidelines will be followed at all times.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through our extensive network of key opinion leaders, partners and collaborators, I will be kept informed and up-to-date of all the latest developments in the screening and assessment of gene delivery systems in vitro and in vivo. Beyond this network, I will frequently attend academic and industry symposia/conferences/talks that will alert me to any possible refinements to current LNP discovery and development pipelines. I am also signed up to newsletters and alerts from organisations dedicated to helping the global research community to identify, develop and implement 3Rs technologies and approaches within their discover pipelines. These organisations include the NC3Rs (https://www.nc3rs.org.uk), Alternatives to Animal Testing (https://caat.jhsph.edu) and Norecopa (https://norecopa.no). I will also ensure to keep informed of any updated guidelines provided by the US Nanoparticle Characterisation Laboratory. Any validated/recommended development that enables the reduction, replacement or refinement of animal experiments will be implemented quickly within our LNP-NA discovery pipeline.

112. Rodent Imaging for Translational Research

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

imaging, therapy, drug, disease

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to provide our partners high quality imaging data to aid decision making on the progression of their projects.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

A new drug is unlike anything used before, researchers may not be quite sure what type of specialist scan to use, or how to interpret any changes in the scan after the drug is given. We will use similar types of scan to look at the new drug. We will use our scanning results to advise medical researchers how best to scan the human patients. Our scanning results will also help the researchers understand the changes they see in the scans of human patients. This is called “translational research”.

We will use our specialist scanners to understand how such substances distribute around the body, and we will try to improve the scanning techniques.

What outputs do you think you will see at the end of this project?

Our imaging studies will play a part in making new medicines available for doctors to prescribe.

Sometimes the ideas for new medicines that come out of the lab are just not good enough to make a worthwhile new medicine. We will help stop those projects as soon as we can by providing information to enable researchers to make decisions on their projects.

We have a lot of experience with rodent scanning, and how to avoid pain and distress. We will work hard to share our methods and ideas with others, so they make the very best use of animal scanning. This will be done by presenting our work at conferences and events and sharing our ideas with our partners widely.

Who or what will benefit from these outputs, and how?

The drugs we test will be at different stages of development some of the outputs and impacts we will provide will be in the short-term others much longer.

Short term outputs and impacts include providing information to our partners to move forwards or stop their project prior to the next stage of development.

Medium term outputs and impacts include generating a data package to provide evidence to funders and regulators to provide evidence that the drug is working.

Long term outputs and impacts include the drug we have tested and imaged going into the patient.

How will you look to maximise the outputs of this work?

We collaborate with a wide range of partners across the UK. We actively work to publish and disseminate our imaging research through conference attendance, publications, case studies on our website.

When engaging with our partners we use the knowledge we have gained through the generation of other imaging studies to help guide the most refined and appropriate study design to meet their needs.

Species and numbers of animals expected to be used

- Mice: 2500
- Rats: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are going to use adult rodents in the course of this project licence. This includes mice and rats.

Adult mice and rats will be used as there are several available mouse and rat models of the diseases we will image.

The models we will use in our imaging studies will mirror the model development performed in other translational studies of this nature.

Typically, what will be done to an animal used in your project?

Typically the animals used in this project will:

1) Receive therapeutic administration of an investigational substance.

2) Be anaesthetised in preparation for their imaging procedure.

3) Receive contrast agent for their imaging procedure.

4) Have a temperature probe in place to monitor their temperature during imaging.

They may:

1) Require blood samples to be taken.

2) Require surgery and cannulation in order to receive the drug or contrast agent.

3) Require tumour cells or other disease models to be implanted and injected.

4) Require multiple imaging sessions and be imaged on multiple days for a period of time.

Animals will then be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

Sometimes the investigational substance may cause the animal to lose weight. We will monitor the weight of the animal closely. We give them an anaesthetic to stop them moving. Animals are expected to make a rapid recovery from their anaesthetic.

We may need to take a blood sample to assist in our understanding that the drug is working. This should only cause pain for a short time.

For cancer medicines we may inject some cells under the skin which grow to form a lump (tumour) under the skin, which we can image. Although this looks unpleasant it doesn't cause the mice much distress. We will monitor the size of the tumour and ensure that the animal can move easily.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity on this licence is >90% of protocols undergoing moderate severity. This is due to the tumour implantation step and also the use of anaesthesia. We have classified the use of anaesthesia as moderate due to the ability to be able to perform repeated longitudinal repeated imaging. One protocol is mild this is due to being a terminal anaesthetic step.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The science of drugs and how the body will act upon administration is not reliably predictable from cell based research.

In translational research, the principal opportunity to avoid the use of animals is simply to progress straight from cell based research to man.

There may be some circumstances where physicians can design clinical trials of investigational therapies using imaging, purely on the basis of existing data, without demanding new animal experiments.

However in clinical trials of the majority of innovative and new medicines, any prior human literature is either of limited value or not available and animal usage and animal research is essential.

In addition these experiments and assessment are required as a dynamic and living assessment and therefore, at present the use of animals is essential.

Which non-animal alternatives did you consider for use in this project?

We regularly review the scientific literature to ensure that we can respond to new developments in model design particularly where newly emerging cell based research and organ on a chip techniques could replace animal use.

Within our organisation we have a complex cell model function which we regularly engage as to whether appropriate cell based screens or organ on a chip methods are an option. Ultimately the final assessment and validation will be required in-vivo to perform a validation of these.

We attend regular internal meetings where we share research outputs with our peers. We engage regularly and have on-going collaborative relationships with the NC3R. We attend conferences on a regular basis and have a national network of partners.

Methodological protocols wherever possible will be performed on non-animal alternatives, e.g. fruit, vegetables, phantoms and rodent cadavers.

Why were they not suitable?

The use of complex cell models offers exciting potential however the correlation between cell based research and patient data within clinical trials remains a major concern

Cell based screens whilst increasing momentum in their development and moving into 3-Dimensional read outs still lacks the physiological requirements of the live body that is needed to understand if the drug is working in the living organism.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have assessed the usage of animals on our current imaging project licence Rodent Imaging for Translational Research. Over the course of 5 years this is a use of 500 mice in one year and 300 rats.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A. In the design of experiments, a stepwise approach will be used, starting with lowest-risk, least- invasive and lowest-number experiments first, e.g. growth curves and tolerability studies in small numbers preceding definitive imaging studies. Typically, non-recovery studies will occur before recovery studies.

B. Our extensive experience and a knowledge of the reproducibility of each imaging modality and predicted effect facilitates effective sample-size calculations.

C. We use (as appropriate) experimental designs such as prespecified size and age ranges for tumours; randomisation according to time and day or tracer uptake; prespecified data quality inclusion criteria and blinding at acquisition and analysis.

D. Designing studies that allows for modification after study start.

E. Where appropriate imaging data will be generated to create predictive models on which drug dose will work.

F. Imaging offers a major reduction over traditional approaches such as histopathology.

G. Our experimental design strategy is informed by use of our in-house powering software.

Where necessary we have and will use the services of an expert statistician to provide additional guidance.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where information is not previously available, we will perform pilot studies to understanding the variability and reproducibility of the data. Within our organisation we will share tissue wherever possible and on a site wide basis and maximise the amount of tissue collection and information that we can derive from each study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This majority of studies in this project will be using either naïve animals or models which reflect conditions seen in the clinical situation. All these studies are designed to have the shortest duration and lowest doses of therapeutic agent that allow for effectiveness of treatments to be assessed. The severity experienced by the animals is managed with observations at a frequency appropriate for the model. Genetically altered animals that demonstrate no or only Mild signs will be used.

We will use mice and rats know as the lowest order of animals that provide suitable data to allow decision to be made. It can be unpleasant for the rodents when they come around from anaesthetic and we need to watch them carefully and keep them warm. Some of the potential new medicines might be unpleasant or even harmful and we follow a series of published guidelines written by vets to ensure we can pick up if an animal is suffering and step in quickly.

Imaging and the ability to image animals longitudinally provides a method of obtaining the information with less animals than historically required e.g., histology.

Why can’t you use animals that are less sentient?

We will wherever possible use terminal anaesthesia. Non-recovery studies would occur before recovery where possible. The use of adult mice is required as it mirrors the model development in other translational studies of this nature.

Options such Zebrafish, Drosophila and Galleria are not feasible as they lack the translational outputs required to move through pre-clinical assessment.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We mainly use mice and rats since they have been used a lot in the past by other researchers, and we can build on what they know.

After any procedural intervention we will closely monitor the animal and seek ways where appropriate to offer a course of pain management. We follow veterinary advice and recommendations on refined handling methods.

We will regularly critically appraise what we do to seek out any ways to improve our models to reduce harm to animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We work closely with current published guidelines and will actively look to collaborate with partners to maintain best practice throughout. We work closely with our named persons and veterinary support to quickly identify and review new guidelines as published.

Guidelines include https://www.nc3rs.org.uk/experimental-design-assistant-eda and https://journals.sagepub.com/doi/full/10.1177/0023677217724823. Imaging specific guidelines include Reporting Guidelines for Imaging Research - ScienceDirect . Evaluating tumor response with FDG PET: updates on PERCIST, comparison with EORTC criteria and clues to future developments - PubMed (nih.gov)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We work closely with the NC3R's to actively identify and pursue new advances as they arise. Our named persons and veterinary support work with us to promote the use of 3R's at all times. We receive literature and are registered with bodies to promote and disseminate such advances to us. We investigate and discuss with our project partners and actively collaborate and assist where possible in the implementation of novel advances.

113. Mechanisms of Brain Development, Function and Ageing

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Neurodevelopment disorders, Cognition, Ageing, Genetics, Environment

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to understand how genetic and environmental changes affect brain development, function and ageing. Gene mutations and environmental exposures associated with brain disorders such as autism spectrum disorders (ASD), intellectual disability (ID) and advanced or reduced cognitive decline with ageing will be investigated to understand the fundamental mechanisms responsible for these conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The identification of the mechanisms that control brain development and brain function and are responsible for specific brain disorders and cognitive decline with ageing will represent significant scientific advances, which will improve diagnosis and may allow preventative treatments to be started early. In the longer-term, these findings are expected to result in treatments for specific conditions, which include autism, intellectual disability, and age-related memory deficits with or without neurodegeneration.

What outputs do you think you will see at the end of this project?

Knowledge: This project will generate insights into the fundamental mechanisms that control brain development, learning and memory and brain ageing. We will identify the causes of specific developmental defects associated with a range of human syndromes associated with ASD and ID, identify fundamental mechanisms by which memories are formed and stored in the brain and identify the changes associated with brain ageing leading to changes in how memories are formed, stored and recalled.

Publications and dissemination: We will continue to publish our results in peer-reviewed, open access leading scientific journals in the field. We will continue to engage with patient and family groups affected by these conditions.

Products: We will aim to translate our knowledge of fundamental mechanisms in pre-clinical studies to identify interventions that can prevent or treat these conditions.

Who or what will benefit from these outputs, and how?

In the short-term, this work will benefit other researchers, clinicians, patients and families by increasing our understanding of these conditions. In the medium- to longer-term, our findings may be translated for direct clinical benefit.

How will you look to maximise the outputs of this work?

Publications will be in open-access journals to ensure unrestricted access. Throughout this project we will collaborate with a number of world-leading scientists and clinicians in

different fields, which include human geneticists, neurologists, experts in learning and memory and ageing. Our work will be presented at both national and international scientific meetings and patient-led groups e.g. the CHARGE syndrome foundation. We will continue publishing and disseminating negative findings as well as unsuccessful approaches. Our research forms the basis of undergraduate and postgraduate teaching ensuring that the next generation of scientists and medical graduates benefit directly from this work.

Species and numbers of animals expected to be used

Mice: 32500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice will be used to take advantage of the well-established protocols for generating genetic models of disease, including the ability to develop tissue- and cell-type-specific mutants and manipulating gene expression in specific brain regions. The mouse allows the analysis of cellular processes and the ability to study the impact of these manipulations on specific behaviours and learning. The mouse is by far the most versatile genetic model system for modelling genetic disease and development of the mammalian brain. Optimised methods are available for all the analyses we intend to perform during the course of our studies. The mouse allows us to study the brain throughout the entire life course, from early embryonic development, through the development of normal brain function in postnatal and adult animals, to the decline of brain function during ageing.

Typically, what will be done to an animal used in your project?

For developmental brain studies, most adult animals used in this project will be maintained under the breeding protocols for generating embryos and postnatal animals for experiments. Thus, mice used will be subject to minimal stress, many of the experiments will involve only breeding and maintenance of mice, followed by killing to obtain tissues. In some cases it is necessary to administer agents to the animal (or to the pregnant dam) in order to label or identify cells and tissues, or to affect gene expression of inducible genes. This will involve no more than injection or feeding of animals, often on one occasion only. Similarly, alterations in the environment to be used in this project have been tested and shown to cause minimal stress to animals with no significant impact on animal health and well- being.

The most invasive techniques proposed in this project involves surgery to introduce compounds or viruses via a small pin-hole in the skull. These studies constitute a relatively small part of the work undertaken with <500 animals undergoing surgery.

Behavioural tests that can cause moderate stress to the animals like fear conditioning and Morris water maze will make use of optimised, refined procedures shown to yield robust data with minimal levels of stress to the animals. These methods have been refined under our previous PPLs and we will continue to refine these methods to reduce stress to the animals.

In some cases, we may kill animals by a non-Schedule 1 method. This may include decapitation of pups, up to the age of 10 days, or we may kill by perfusion and/or fixation under terminal anaesthesia, in order to optimize fixation of tissues prior for subsequent laboratory analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

1) Surgery may result in post-operative pain that will be temporary and will be controlled by appropriate analgesics.

2) Ageing is associated with increased incidence of conditions like cancer, skin irritation and weight loss.

3) Certain genetic conditions we model is associated with a range of abnormalities, including craniofacial and tooth abnormalities that may affect feeding, leading to weight loss.

4) Some genetic conditions may increase chances of seizures, these are not expected to last more than a few minutes and mice will be killed if they had more than one seizure.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Referring to the categories above:

1) Mild, <1%

2) Moderate, ca. 20%

3) Moderate, ca. 20%

4) Moderate, <2%

What will happen to animals at the end of this project?

- Killed

- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Brain development is a complex process that are orchestrated by the communication of several different tissue types and often involves the migration of cells or extension of cell processes (such as axons) over long distances. These complex processes cannot be modelled fully in vitro. Psychiatric disorders such as autism are characterised by specific combinations of behavioural traits that are impossible to study in vitro. Memories are stored in sparsely distributed, interconnected networks of neurons in different brain areas; the study of learning and memory therefore requires animal studies. Age-related cognitive decline is to some extent regulated by other systems in the body, most notably the immune system and the interaction between immune factors and the brain requires intact animals. The experiments we are proposing relate to function in the conscious behaving animal for which there is no suitable alternative. It is not ethical to manipulate either the genetics or the environment, especially during early development, in humans. Therefore, there is no feasible alternative that would entirely replace animals.

Which non-animal alternatives did you consider for use in this project?

Where possible, certain aspects of the phenotypes will be studied and modelled in cell lines. For example, we have access to human hippocampal and cancer cell lines that can recapitulate some aspects of neurogenesis and cell behaviour in vitro. We are establishing collaborations that will allow us to assess the roles of some of these genetic factors in early stages of human brain development using human ES and iPS cell-derived brain organoids and neurons integrated into newly developed in vitro circuits. Short-term ex vivo experiments (such as analysing cell proliferation, differentiation, migration and electrophysiological properties in organotypic slice cultures) will be employed where possible (e.g. where we are only interested in a short developmental time window).

Why were they not suitable?

The approaches above can be useful for studying single isolated cell types and neuronal development at early developmental stages. However, we need to investigate complex systems that develop over a long time and brain functions that are regulated by many different cell types, distributed through the brain and body of the organisms. Furthermore, we need to study complex behaviours over the life time which is not possible without studying animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Number of animals are estimated based on experience from previous PPLs and research publications from my group, collaborators and external experts.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The majority of experiments will involve the breeding of GA animals for timed-mated embryos or postnatal animals. Wherever possible, we produce mouse lines that are homozygous for conditional alleles. This allows us to significantly reduce the number of mice needed by increasing the Mendelian ratio of homozygous mutants from 1:4 to 1:2 for a single gene and from 1:16 to 1:4 for two genes.

Apart from experiments in early embryos where only one experimental read-out can be obtained per embryo due to their small size, experiments at later stages are designed such that multiple parameters are assessed per sample. For example, mice are injected with BrdU that allows visualisation of proliferating cells in sections. Adjacent sections can be used for standard histology, in situ hybridisation and immunohistochemistry. Where possible, multiple antibodies are used per section to increase the amount of information obtained even further. Statistical analyses are done to ensure the minimum number of animals are used to obtain significant data. Upon completion of behavioural assays, brains are collected for ex vivo MRI studies, so that a separate cohort of animals does not need to be bred for this purpose.

We will follow best practise for breeding and colony management, and archive GA mice where possible, as recommended by NC3Rs:

https://nc3rs.org.uk/3rs-resources/breeding-and-colony-management/sharing-and-archiving-ga-mice https://nc3rs.org.uk/3rs-resources/breeding-and-colony-management

Unnecessary production or import of GA mouse lines will be avoided by searching databases, including:

https://mouse-locator.crick.ac.uk/ Pubmed: http://www.ncbi.nlm.nih.gov

Jackson laboratory: http://www.informatics.jax.org

Strains produced by us are entered into the Jackson laboratory MGI database, cryopreserved and made available to other investigators via shared repositories such as EMMA and MMRRC.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding strategies are optimised for each line to ensure best possible Mendelian ratios. Apart from experiments in early embryos where only one experimental read-out can be obtained per embryo due to their small size, experiments at later stages are designed such that multiple parameters are assessed per sample. For example, mice are injected with agents that allows visualisation of dividing cells in tissue sections. Adjacent sections can be used for other analyses, increasing the amount of information that can be obtained per animal. Upon completion of behavioural assays, brains are collected for imaging studies, so that a separate cohort of animals does not need to be bred for this purpose.

Optimal group sizes are determined based on previous data or, if not available, by pilot studies to ensure experiments are not under- or over-powered.

Many of the genetic conditions we study model complex human syndromes, characterised by additional phenotypes in addition to neurodevelopmental features. We collaborate with other development groups including those working on deafness and craniofacial anomalies, where we share tissues from relevant mutants with these groups for analysis.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically modified mice will be used to take advantage of the well-established protocols for generating tissue- and cell-type-specific mutants, analysing cellular processes during brain development and behavioural analysis. The mouse is by far the best genetic model system for modelling genetic disease and development of the mammalian brain. Optimised methods are available for all the analyses we intend to perform during the course of our studies.

Most adult animals used in this project will be maintained under the breeding protocols for generating embryos and postnatal animals for experiments. Thus, mice used will be

subject to minimal stress, many of the experiments will involve only breeding and maintenance of mice, followed by killing to obtain tissues. In some cases it is necessary to administer agents to the animal (or to the pregnant dam) in order to label or identify cells and tissues, or to affect gene expression of inducible genes, this will involve no more than injection or feeding of animals, often on one occasion only. Similarly, alterations in the environment to be used in this project have been tested and shown to cause minimal stress to animals with no significant impact on animal health and well-being.

Behavioural tests that can cause moderate stress to the animals like fear conditioning and Morris water maze will make use of optimised, refined procedures shown to yield robust data with minimal levels of stress to the animals. These methods have been refined under our previous PPLs and any pain or stress are transient. We continue to refine methods and attempt to complement and them with approaches that are less stressful for the animals when possible.

In some cases, we may kill animals by a non-Schedule 1 method. This may include decapitation of pups, up to the age of 10 days, or we may kill by perfusion and/or fixation under terminal anaesthesia, in order to optimize fixation of tissues prior to subsequent laboratory analysis. In some cases, we may harvest blood or tissues under terminal anaesthesia. Depth of anaesthesia will be monitored at all times to ensure that the animals suffer no pain, suffering or distress.

Recovery surgery is required to introduce substances into specific brain areas. These are relatively minor surgeries, post-operative pain is transient and will be managed with appropriate analgesia.

None of the methods to be used in this project are expected to cause lasting pain, suffering or distress to the animals.

Why can’t you use animals that are less sentient?

Our aim is to model human conditions and as such, a mammalian system that share the same genes, similar brain structure and functional properties is essential. Complex behaviours associated with psychiatric conditions and intellectual disabilities that rely on multiple brain systems cannot be adequately studied in less sentient organisms like zebrafish or Drosophila.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All surgery will be conducted using aseptic technique which meets at least the standards set out in the HO Minimum Standards for Aseptic Surgery. Consequently, postoperative infections are not expected. Animals are expected to recover uneventfully from anaesthesia and surgery. Appropriate analgesia will be provided post-surgery.

For stereotactic surgeries, the small size of the incisions, the fine pipettes used, and the small volumes injected into the brain tissue limit the risk of mechanical brain damage. Although unlikely (not observed in the past 5 years of similar work) if damage occurs, it will only affect a small region of the brain at the site of injection. To ensure that no behavioural alterations develop after surgery, animals will be monitored daily for any deviation from normal behaviour. If there is any deviation from normal behaviour they will be killed by a schedule 1 method.

Mice displaying body weight loss of greater than 10% but less than 15% body weight will receive moist macerated diet at floor height to enable easy access to food and hydration. If male mice are to be group housed, only siblings will be housed together to minimise fighting. All new diets will be compared for palatability relative to standard (control) diets in a pilot study and palatability can be modified for example with the use of artificial sweeteners (e.g. Sucralose ©). Any bleeding after blood sampling will be stopped by local pressure.

To ensure robust assessment of learning behaviours, animals are handled for 5-6 days and acclimatised to test areas where possible and where it will not interfere with the actual test.

For stressful behavioural tests like Morris water maze and Contextual fear conditioning, we will use optimised experimental procedures to ensure robust learning, thus reducing the numbers of animals needed to obtain robust results, whilst minimising unnecessary stress to the animals as much as possible.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The most up-to-date, optimised and refined approaches will be used from publications in the field. We will consult with our N3CRs programme manager for specific advice where appropriate.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institution has a dedicated NC3Rs Regional Programme Manager, who provides dedicated 3Rs advice and support at project, laboratory and institute levels. This includes assisting in identifying new 3Rs opportunities and coordinating the sharing of best practice across the different sites. The manager attends our regular animal users committee and keeps us up to date with new developments and how to effectively incorporate these in our work.

114. Understanding and Modulating the Crosstalk Between the Heart and the Immune System

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

immunopathology, autoimmunity, heart failure, myocardial infarction

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this program of work is to characterise the crosstalk between the immune system and the heart and to identify ways to prevent immune-mediated heart tissue damage.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There are currently over half a million people in the UK living with heart failure. Improved intervention techniques have significantly increased immediate survival after a heart attack, also called myocardial infarction (MI). However, an MI causes the death of heart muscle cells (cardiomyocytes) and destruction of possibly large areas of tissue due to an acute lack of oxygen. As the regenerative capacity of the adult human heart is minimal, injury to the heart muscle frequently initiates the development towards heart failure, which defines a functional deterioration until the heart is too weak to pump sufficient blood around the body.

The immune response to heart tissue damage has been suggested as a crucial contributing factor to deterioration after MI as well as other heart conditions. Importantly, patients with immunological diseases including rheumatoid arthritis or systemic lupus erythematosus, have a significantly worse prognosis after MI as well as a higher risk baseline cardiac risk compared to the general population, which has been attributed to systemic inflammation.

Current heart failure therapy cannot restore normal heart function and the major goal is to prolong the patient’s life and reduce symptoms. Therefore, new strategies to improve repair and prevent heart failure remain a major therapeutic goal in cardiology. We believe that targeting the immune response against the heart needs to be a critical component of such strategies.

What outputs do you think you will see at the end of this project?

The program of work covered by this project licence will contribute to global efforts aiming to understand and provide new treatments for immunopathology in heart disease. Outputs will largely be new information presented in form of scientific publications, with an outlook towards translation into clinics.

1. An improved understanding of the processes triggering immunopathology in the heart: As a short/mid-term benefit of this project, we expect to generate valuable data of interest to the research community of both pre-clinical and clinical scientists. The proposed study bridges immunology, cardiology and regenerative medicine and is at the forefront of a newly emerging field of research. It will advance our knowledge of how the immune system affects the heart and what aspects of the immune response to tissue injury are beneficial versus detrimental.

2. The identification of new therapeutic targets for efficient modulation of the pathological immune response against the heart: The long-term benefits of this project will be the identification of new target pathways and therapeutic options to improve heart regeneration and treat inflammatory conditions affecting the heart.

Who or what will benefit from these outputs, and how?

1) Basic and clinical scientific community: Progress under this project licence will be of significant interest to the cardiovascular research community. Cardio-immunology has recently emerged as a research field of significant translational relevance and the program of work covered here will contribute important results of global scientific interest. This will be realised through publications in scientific journals and dissemination of results through international conferences. The immediate beneficiaries of this work will thus be scientists including basic researchers and clinical colleagues.

2) Patients: In the long-term, animal data is essential to allow new therapeutic targets to be taken forward to clinical trials which will be the prerequisite for patient benefit. Improving quality of life and a positive impact on patient morbidity and mortality through immuno-modulation is the ultimate long-term goal of this program of work.

Notably, results obtained from this study will be applicable to other settings, where aberrant immune responses are the underlying cause of tissue destruction. By investigating the possibility of inducing tolerance against target organs of immune-mediated damage, the proposed study therefore addresses several clinically relevant issues and has high potential for wider clinical impact beyond the cardiovascular setting.

How will you look to maximise the outputs of this work?

We are embedded in a wide collaborative network of basic and clinical scientists and clinicians, both locally and internationally, which will support dissemination of our work via the traditional scientific route, i.e. meetings, conferences and publications.

Depending on the type of information to be shared, seminars and workshops may be useful tools. We also use a range of dissemination channels besides traditional scientific conferences and journals, including less formal outlets such as social media platforms and participation in public events for outreach to the wider community.

Species and numbers of animals expected to be used

- Mice: 8000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We have chosen to use mice as a suitable model species for this exploratory pre-clinical investigation. Mice are universally used for work involving genetic alterations. Standard protocols, methods and reagents have been optimised for mice. For experimental protocols we will use adult mice from 8 weeks of age, as the immune system is considered mature from this age onwards.

The proposed research involves the investigation of complex multi-dimensional processes and evaluation of organ function in crosstalk with the immune system. It is unfortunately still impossible to produce a meaningful in vitro replica of the complete system of molecular, cellular, physiological, and behavioural interactions influencing each other during complex in vivo processes. Thus, there is to- date no non-animal replacement and rodent models are an essential and recognised pre-clinical step.

The use of lower vertebrate species (flies/fish) will not be adequate for this project, as a mammalian model system is key to understanding human physiology and disease. From a neurophysiological perspective, the mouse is considered among the least sentient mammalian species, while still sharing fundamental physiological and pathophysiological features with humans. Importantly, they can be easily genetically altered to allow assessment for individual pathways and a wide range of GA lines are available already.

Typically, what will be done to an animal used in your project?

1) Mice in heart-injury studies will typically be treated by a single ('high-dose') intraperitoneal injection of a cardiotoxic agent (e.g. adrenergic agonist) to induce acute heart injury. Rarely, mice may receive repeated intraperitoneal injections or the implantation of an osmotic minipump to deliver the cardiotoxic agent ('low-dose') over time.

In less than 5% of mice, pre-treatment may include bone marrow transplantation following irradiation. This will involve irradiation and one intravenous injection. This allows to analyse the role of a gene of interest specifically in immune cells.

After induction of cardiac damage, mice will generally be monitored for a maximum of 8 weeks, including blood sampling and imaging. Blood sampling (~50% of total experimental animals) will involve the puncture of a superficial vein (e.g. saphenous vein) and collection of a blood sample for a maximum of 6 times over the lifetime of an animal. In vivo imaging will be performed under general anaesthesia (~50% of total experimental animals), and most animals will only undergo brief echocardiography sessions (maximum of 6 recovery sessions and 1 non-recovery session total over lifetime). For only a few selected animals 2 of these 6 sessions may be replaced by specialised imaging modalities, such as MRI or PET/SPECT.

Either before or after induction of cardiac damage, ~50% of total experimental animals will be injected by the intravenous or intraperitoneal route by experimental (e.g. for transgene

induction) and/or therapeutic substances (e.g. immunomodulatory agents) at a maximum of 8 total treatments over the lifetime of the animal.

The anticipated typical animal experience will be a single intraperitoneal injection of a cardiotoxic agent, followed by administration of an immunomodulatory agent (subcutaneous, intravenous or intraperitoneal route), longitudinal blood sampling (in two-week intervals for a maximum of 8 weeks) and echocardiography imaging (in two-week intervals for a maximum of 8 weeks).

2) Mice in inflammatory studies will typically be treated by topical application of an inflammatory agent to the skin to induce systemic inflammation/autoimmunity. This will be performed 3 times weekly, for a maximum duration of 4 weeks.

In less than 5% of mice, pre-treatment may include bone marrow transplantation following irradiation. This will involve irradiation and one intravenous injection to allow analysis of the role of a gene of interest specifically in immune cells. Some animals may receive a high fat diet to mimic atherosclerosis-like inflammatory effects.

After induction of systemic inflammation/autoimmunity, mice will generally be monitored for a maximum of 12 weeks, including blood sampling and imaging. Blood sampling (~50% of total experimental animals) will involve the puncture of a superficial vein (e.g. saphenous vein) and collection of a blood sample for a maximum of 6 times over the lifetime of an animal. In vivo imaging will be performed under general anaesthesia (~50% of total experimental animals), and most animals will only undergo brief echocardiography sessions (maximum of 6 total over lifetime). For only a few selected animals 2 of these 6 sessions may be replaced by specialised imaging modalities, such as MRI or PET/SPECT.

Either before or after induction of systemic inflammation/autoimmunity, ~50% of total experimental animals will be injected by the intravenous or intraperitoneal route by experimental (e.g. for transgene induction) and/or therapeutic substances (e.g. immunomodulatory agents) at a maximum of 8 total treatments over the lifetime of the animal.

The anticipated typical animal experience will be non-invasive topical application of an inflammatory agent to the skin 3 times a week for a maximum duration of 4 weeks, followed/accompanied by administration of an immunomodulatory agent (subcutaneous, intravenous or intraperitoneal route), longitudinal blood sampling (in two-week intervals for a maximum of 12 weeks) and echocardiography imaging (in at least two-week intervals for a maximum of 12 weeks).

3) Mice in combined studies will first receive an inflammatory agent as described in 2). After full recovery from acute inflammation, heart injury will be induced as described in 1).

In less than 5% of mice, pre-treatment may include bone marrow transplantation following irradiation. This will involve irradiation and one intravenous injection to allow analysis of the role of a gene of interest specifically in immune cells.

After induction of systemic inflammation/autoimmunity and cardiac damage, mice will generally be monitored for a maximum of 12 weeks, including blood sampling and imaging. Blood sampling (~50% of total experimental animals) will involve the puncture of a superficial vein (e.g. saphenous vein) and collection of a blood sample for a maximum of 6 times over the lifetime of an animal. In vivo imaging will be performed under general anaesthesia (~50% of total experimental animals), and most animals will only undergo brief echocardiography sessions (maximum of 6 total over lifetime). For only a few selected animals 2 of these 6 sessions may be replaced by specialised imaging modalities, such as MRI or PET/SPECT.

Either before or after induction of systemic inflammation/autoimmunity and cardiac damage, ~50% of total experimental animals will be injected by the intravenous or intraperitoneal route by experimental (e.g. for transgene induction) and/or therapeutic substances (e.g. immunomodulatory agents) at a maximum of 8 total treatments over the lifetime of the animal.

The anticipated typical animal experience will be the non-invasive topical application of an inflammatory agent to the skin 3 times a week for a maximum duration of 4 weeks and induction of cardiac damage by a single intraperitoneal injection of a cardiotoxic agent, followed/accompanied by administration of an immunomodulatory agent (subcutaneous, intravenous or intraperitoneal route), longitudinal blood sampling (in two-week intervals for a maximum of 12 weeks) and echocardiography imaging (in at least two-week intervals for a maximum of 12 weeks).

In the end, mice will be humanely killed and tissues/organs collected for further ex vivo analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

As we are inducing heart disease and systemic inflammation, adverse effects may occur.

1) Mice receiving treatment to induce myocardial injury (single high dose) show apparent clinical signs for the first few hours after treatment, which are expected to resolve within 24h. Clinical signs include an increased respiratory rate and lethargy, which are expected manifestations of a high cardiac workload. An acute mortality of around 1% has been observed previously in the first 48h due to heart complications which are most likely arrhythmic and occur as instant cardiac death. Once recovered from acute effects, we do not expect any further adverse effects as any chronic heart failure phenotype is mild and expected to remain subclinical in this model. We do also not expect any adverse effects of low dose protocols.

2) Mice receiving treatment to induce inflammation may develop signs of illness such as reduced mobility, hunching and ruffled fur. Mice in inflammation studies will therefore be monitored closely taking into account appearance and activity. Treatment will immediately stop and monitoring will be increased if the above signs of ill health develop. From previous experience, affected animals recover within 48h after cessation of treatment, but will be humanely killed if not. If a change in diet is included in the protocol, the new diet may not be palatable to some animals, which then fail to eat and lose weight. In rare cases, animals fed high fat diets may develop dermatitis or obesity.

Common optional treatments that may cause pain or distress include additional injections for bone marrow transplantation, anaesthesia for in vivo imaging, treatment with experimental or therapeutic substances and punctures for blood sampling.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Based on previous retrospective severity scoring, the experience of mice is expected to be 25% sub- threshold, 25% mild and 50% moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The proposed research involves the investigation of complex multi-dimensional processes and evaluation of organ function in crosstalk with the immune system. It is unfortunately still impossible to produce a meaningful in vitro replica of the complete system of molecular, cellular, physiological, and behavioural interactions influencing each other during complex in vivo processes. Thus, there is to- date no non-animal replacement and rodent models are an essential and recognised pre-clinical step.

Which non-animal alternatives did you consider for use in this project?

Animal work will be supported by a variety of in vitro and ex vivo approaches. Cell-specific functional and mechanistic studies will be performed ex vivo or in vitro. Examples are the investigation of changes in cellular phenotype and function in response to treatment with

post-injury serum. These assays will be performed on cell lines or ex vivo cells from mice killed by a schedule 1 method.

In addition, a protocol of analysing tissue morphology in thick cardiac slices has been set up in the Section, which will allow replacement of some in vivo imaging sessions using contrast agent administration for the assessment of fibrosis.

We are further expanding the use of human tissue; cardiomyocytes derived from human induced pluripotent stem cells will be used to study patient serum and immune cells. The PPL holder is also a member of the 'Human Tissue User Group' who hold the relevant ethics to receive rejected donor and diseased hearts.

Why were they not suitable?

The use of lower vertebrate species (flies/fish) will not be appropriate for this project, as a mammalian model system is key to understanding human physiology and disease. Fish have the capacity to regenerate their hearts and do not develop heart failure as mammals do. It is also still unknown if they develop autoimmunity. While it may be of great scientific interest to compare heart disease and autoimmunity between fish and mammalian species, the research question posed here can unfortunately not yet be answered using fish.

From a neurophysiological perspective, the mouse is considered among the least sentient mammalian species, while still sharing fundamental physiological and pathophysiological features with humans.

Importantly, they can be easily genetically altered to allow assessment for individual pathways and a wide range of GA lines are available already. Embryonic forms are not suitable due to their immature immune system.

A range of non-animal alternatives are used as described above. While they are able to provide valuable information on selected pathways and can be used for confirmation of individual mechanisms, they are not suitable to study the in vivo pathological effects of immune system aberrations on the heart and vice versa.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated experimental mouse numbers used in this project based on the anticipated number of experiments, experimental groups, and number of animals per group. These figures are based on our own and collaborators' previous experience.

The number most difficult to estimate is the breeding number as it will depend on several factors including specific strains used, litter sizes and GA breeding strategy. Our estimate assumes the use of both males and females as well as the use of wildtype littermates as controls.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The experiments proposed in this licence are primarily based on procedures we have longstanding experience with, which allows us to base experimental design considerations on a solid base of prior data. We will continue to design and perform experiments following general principles of good experimental design and laboratory practice. Our in-house Statistical Advisory Service may be consulted if necessary. We routinely use G*Power software for Power analysis and GraphPad Prism for statistical analysis. To avoid bias and variability, experiment al design will include randomisation, blinded assessment, and explicit inclusion and exclusion criteria.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Experiments will preferably follow a longitudinal approach to reduce necessary numbers due to inter- individual variation at baseline and maximise output from individual mice. This will apply specifically for immunological measurements known to vary between individuals and in vivo imaging experiments.

Breeding of GA lines will follow guidelines to avoid overproduction and surplus animals. Both male and female mice will be used for our protocols and ‘wrong genotype’ animals used as controls or for tissue collections.

To minimise the number of necessary repeats, individual project aims covered by this licence will be performed in parallel by several researchers to assess a variety of structural, functional and metabolic parameters. Data obtained from control group animals that do not undergo any experimental procedures besides monitoring may be shared between experiments.

Being embedded in a cardiovascular environment, we have established a system of collecting spare lymphoid organs from experimental animals that undergo MI surgery for unrelated projects. This has already reduced the number of additional surgical procedures significantly.

We adhere to established standards of experimental design to allow reporting according to the NC3R ARRIVE guidelines, and optimise number of readouts obtained from each procedure

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All proposed procedures will be performed under appropriate anaesthesia and/or analgesia to minimise stress and suffering as appropriate. All proposed models and methods chosen are current state-of-the-art standard for their respective type of work.

The proposed model of systemic inflammation has been optimised to ensure weight-dependent dosing and the shortest possible treatment regime. To ensure accurate dosing and avoid spillage into the eye and/or oral uptake due to licking the ear immediately after application, a very brief general anaesthesia (<5 minutes) for restraint is used. Involvement of the heart in this model has also been thoroughly characterised. We may include genetic models, such as MRL/lpr, BXSB or genetically- modified mouse lines. Using these and/or GA lines (e.g. genetic deletion of a gene in a specific cell type) presents a refinement and will reduce necessary animal numbers.

The protocol for inducing acute cardiac damage by intraperitoneal injection of a one-off dose of the cardiotoxic agent Isoproterenol to induce acute death of heart muscle cells has also been characterised thoroughly. This model is a significantly refined alternative to invasive surgical induction of myocardial infarction. This simple procedure allows to obtain all immunological readouts required for the current program of work without the need of invasive surgery.

In any of our studies, mice may further undergo additional optional procedures (bone marrow transplantation following irradiation, injections of substances, imaging under general anaesthesia,

blood sampling) for pre-treatment, assessment of therapeutic agents and/or monitoring. All procedures are commonly used standard procedures, and we make every effort to use the most refined technique available, including tube handling or blood sampling via the saphenous vein, and longitudinal imaging.

Why can’t you use animals that are less sentient?

The use of lower vertebrate species (flies/fish) will not be adequate for this project, as a mammalian model system is key to understanding human physiology and disease. From a neurophysiological perspective, the mouse is considered among the least sentient of mammalian species, while still sharing fundamental physiological and pathophysiological features with humans. Importantly, they can be easily genetically altered to allow assessment for individual pathways and a wide range of GA lines are available already.

Embryonic mice are also not suitable due to the immaturity of their immune system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our in vivo work is guided by a set of overarching welfare principles to minimise suffering to the absolute minimum necessary, which includes updating procedures if new information becomes available.

(1) Every effort will be made to use the most refined technique available for any procedure. If new information becomes available, we will amend our protocols to ensure most refined approaches.

(2) Ensuring appropriate technical skills and competency of persons involved in animal care and use.

(3) Using appropriate anaesthetic and analgesic regimes for pain relief. General anaesthesia will be used for in vivo imaging and osmotic minipump implantation as well as briefly for topical application to the ear. Osmotic minipump implantation will also require analgesia using a treatment regime discussed with the NVS.

(4) Ensuring that animals are monitored regularly depending on the respective situation. Any cage of animals will be checked daily for appropriate food/water supply and general housing issues. Individual mice will be monitored at least daily for general health. If clinical symptoms develop, monitoring will be further increased together with other supportive measures to ensure humane end points if necessary. After any general anaesthesia, animals will be monitored until having regained consciousness and full mobility.

(5) Humane end points will be defined clearly. In general, clinical/behavioural (e.g. posture, activity) and pathophysiological (e.g. respiration rate, weight loss) readouts will be used to determine humane end points.

(6) General housing conditions will follow best practice to optimise animal welfare and reduce infection risk if necessary, including environmental enrichment and group housing where possible to meet behavioural needs.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow published LASA guidelines as well as the ARRIVE guidelines for reporting of animal studies. We also implement new guidelines when published by the NC3Rs and our local 3Rs advisory group.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Due to being a member of my institution’s animal welfare and ethics committee, I'm in regular close contact with named persons and informed first hand of possible 3Rs advancements. Our team are highly committed to the 3Rs and regularly review our protocols and experimental design in the light of new information. Training on the latest most refined techniques is readily available from the named training and competency officers and the local veterinary surgeons.

115. Molecular Mechanisms Underpinning Neuronal Development and Stress Responses in Rodents

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Stress, Molecular mechanisms, Hippocampus, Behaviour, Brain development

Animal types	Life stages
Mice	juvenile, adult, aged
Rats	pregnant, adult, juvenile, aged, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to advance our fundamental scientific knowledge on the molecular mechanisms in the brain that are essential for brain development and plasticity and, in particular, those underpinning adaptive physiological and behavioural responses to acute and chronic stress.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Stress is a phenomenon of all time but in recent years it has developed globally into a major problem. Stress has been identified as a major causal factor in mental health disorders like major depression, anxiety and post-traumatic stress disorder. The incidence of these disorders has indeed grown to virtually epidemic proportions. It is thought that the ability to respond to stressful events in a way that safeguards well-being (i.e. coping) is critical to maintaining good mental health. Hence, disruption of stress-coping mechanisms is considered a decisive feature of many mental health conditions. These thoughts are, however, still based on concepts but hard scientific evidence substantiating these concepts is lacking. Presently, we still don't know how stress affects processes in our brain, despite the advances in knowledge made during the past decades. Moreover, we still need to learn how changes in our brain occur which help us to cope with stressful challenges in our lives. It's crucial to understand the impact of stress across the life span as the brain goes through distinct phases of development throughout life. It is important to underscore that most neuroscience research has been conducted on male rodents and there is a major gap in knowledge regarding how the female brain responds and adapts to stress. Statistically, the incidence of stress-related mental disorders is much higher in females than in males. One of our principal aims is to gain insight why this is the case. Understanding of how stress impacts on the male and the female brain is critical in order to develop effective therapies for stress-related mental disorders and create preventative measures for both halves of humanity.

What outputs do you think you will see at the end of this project?

The outlined work aims to advance our fundamental scientific knowledge on the molecular mechanisms in the brain that are essential for brain development and plasticity and, in particular, those underpinning adaptive physiological and behavioural responses to acute and chronic stress.

Our work will benefit hugely from our insights into the molecular and cellular responses to stress that occur in the brain collected in recent years.

Specifically, outputs of the planned work will be:

- Elucidation of the molecular processes determining brain development, including the generation of nerve cells ('neurogenesis'), in male and female rodent embryos, neonates, juvenile, adult and aged rodents.

- Identification of the cell types within the male and female rodent brain, including the different stages of neurogenesis, in which these molecular processes are taking place.

- Observations of changes in these molecular and cellular processes under conditions of acute and chronic stress in juvenile and adult rodents. This work should reveal differences in the impact of acute and chronic stress in male and female juvenile and adult rodents and thus show differences in stress sensitivity and stress resilience.

- Observations of changes in these molecular and cellular processes under baseline conditions and acute stress in aged versus adult rodents, possibly revealing that aged rodents may be more sensitive to chronic stress.

- Identification of the key molecular and cellular mechanisms that occur in response to acute stress which underpin the consolidation of adaptive behavioural changes expressed in case a similar stressful event would reoccur. This work is expected to reveal differences in stress responsiveness and behavioural adaptation between juvenile, adult and aged, male and female rodents.

The data generated will be made available through publications in international, open access, peer reviewed journals, conference presentations and proceedings and publicly available next generation sequencing data sets (a significant resource that can be utilised by the entire research community).

Who or what will benefit from these outputs, and how?

Short term benefits (mid-license):

- Detailed novel data on the participating genes and their products (proteins) in embryonic and adult neurogenesis and their response to acute stress. We expect to find differences between juvenile, adult and aged rodents. We anticipate identification of multiple participating genes playing important roles in cellular mechanisms such as cell proliferation, neuronal differentiation, migration and maturation.

- The study of rodent embryonic and neonate brains will show the expression of candidate genes of interest changes at the various developmental stages. These insights will generate additional ideas about the role of these genes (for instance, in responses to acute or chronic stress) in the later life of the rodent.

- We will obtain insight into sex differences in key stress-induced molecular and cellular endpoints.

- Publication of these novel data will be highly valuable for the scientific community.

Medium-term benefits (end of license):

- We expect that the results obtained during the first phase of this project will inform which processes (e.g. genes, protein-protein interactions, cells, cellular interactions) to address as experimental endpoints in its second phase. In this phase, results will be obtained regarding the effects of chronic stress on the molecular and cellular processes in the brain of interest as well as their implications for adaptive and anxiety-related behaviours. We expect to find a clear distinction between molecular, cellular and behavioural observations in stress-resistant and stress-sensitive rodents. These data will enable us to identify the critical genes (5-10?) and cell developmental stage(s) (1-2?) becoming dysfunctional in stress-sensitive rodents after chronic stress. Furthermore, we

will be able then to make a comparative analysis between chronic stress and ageing data which will reveal to what extent the conditions are overlapping in terms of the molecular, cellular and behavioural endpoints under investigation.

- We will have substantially closed the gap in knowledge regarding sex differences in molecular, cellular and behavioural responses to acute and chronic stress.

- If time allows, data will be obtained regarding the molecular (epigenetic?) mechanisms underpinning the aberrant gene and cellular function in aged and chronically stressed rodents.

- We will have established in vitro models (e.g. primary cell cultures) to study the discovered molecular and cellular processes in more details (e.g. signalling and epigenetic pathways, protein- protein interactions, synaptic communication between nerve cells, etc.). This may result in reduction of the number of animals used under a PPL in the future.

- The novel data will be the basis of multiple publications to the benefit of the entire scientific community.

Long term benefits (10-15 years):

- We hope that our discoveries will lead to the identification of candidates for the development of novel drug-based and/or gene therapy based strategies for the treatment of stress-related mental disorders and possibly even age-related mental and/or neurological disorders.

- Our identification of critical genes may initiate a search for gene variants (‘single nucleotide polymorphisms’ (SNPs)) in male and female patient populations hopefully resulting in the discovery of variants that may transmit vulnerability for certain mental disorders. With such knowledge, behavioural therapies and/or life style adjustments may be established to prevent disease development in these subjects. The body of acquired knowledge on the male and female brain will help the development of sex-specific therapies and other measures.

How will you look to maximise the outputs of this work?

Findings and data generated by the outlined studies will be made available to other scientists through publications in international peer-reviewed, and where possible open-access, journals. Presentations of the data will also be made by group members at scientific conferences and meetings and public engagement events.

Species and numbers of animals expected to be used

- Mice: 1160
- Rats: 2840

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rats will be used for the majority of studies as they are the least sentient species that will engage in the tasks eliciting relevant stress responses. Rats have been used extensively in such studies and consequently there is a wealth of core data available on neuronal development and the neurobiology and behavioural effects of stress in this species. Furthermore, previous work has demonstrated a high relevance of findings from stress-related research conducted in rats, for human psychiatric disorders.

Most studies involving genetic manipulations will be conducted in mice due to the technical difficulties of creating and maintaining genetically altered rat lines, thus enabling more sophisticated genetic manipulations to be assessed. The outcomes derived from these mouse studies will provide critical advances in the understanding of the genetic basis of stress responses, albeit for more simplified behavioural outcomes. Where an appropriate rat line is available, carrying the desired genetic modification, it will be used to maximise translatability of research findings and to enable complementary behavioural studies which may be more relevant for the human situation to be undertaken.

Biological responses to stress vary with the developmental stage of an individual. To maximise the scientific relevance of our work it is, therefore, necessary to perform these experiments at different developmental stages (infant, juvenile, adolescent, adult and aged) to more fully understand the molecular identity of each stage and how the stage of development influences the stress response.

Typically, what will be done to an animal used in your project?

Upon arrival in the unit, animals will be left to acclimatise for 7 days, during which time animals will be regularly handled to habituate them to the researchers.

Drug delivery (rats and mice): Approximately half of animals will be given neuromodulatory substances. In most cases either in food or water or by training the animals to voluntarily take the drug mixed in a sweet liquid. In small number of cases (rats only) the drug will be delivered directly into the brain via surgically implanted cannulas.

Surgical preparation (rats only): A small number of rats (~15% total used under license) will undergo a surgical procedure either to implant bilateral cannulas to enable drugs to be delivered directly into the brain or to deliver gene modulating agents (e.g. gene-specific silencing RNA to facilitate long-term knockdown of a specific gene of interest, viral vectors). In a small number of cases (~5% total used under license) implanted cannula

may be linked to a subcutaneously implanted minipump to enable the sustained delivery of a drug directly into the brain.

Animals may then undergo behavioural testing using one of the following scenarios.

Scenario 1 (rats only): The Morris water maze paradigm (or water paddling maze alternative if scientifically relevant) will be used to determine individual differences in stress coping. After an initial acclimatisation/training session of ~3 min duration without a platform, rats will undergo four training trial sessions of ~4 min duration with the platform present to facilitate an active stress-coping response. A maze control group of rats will be exposed to the maze for matched period of time (cf. trained group) but without a platform present to distinguish the stress experience from the active coping component of the response. Both the 'trained' and 'control' groups will include sufficient animals to study individualised responses to the stress, which we can assess in terms of their behaviour (exploratory parameters/strategies). In addition, the 'trained' group will display active stress coping behaviours, namely the ability to find and remember the location of the platform to facilitate early exit from the situation in future exposures, thus providing a measure for the active stress coping component of the response. Molecular pathways which are activated in the 'trained' group that are absent from the 'control' group are therefore likely to be attributed to the active stress coping strategy as opposed to other aspects of the paradigm which are controlled for by the 'control' group (physical time spent in water, trial structure, recovery period, variability of individualised stress responses etc.). All rats will be allowed a ~10 min recovery period in their home cage between each trial. The trial sessions may be repeated, typically daily for 4 consecutive days (max. 7 days). The rat rapidly learns the platform location resulting in very short exposure times in later trials. A final trial session of ~3 min duration may be conducted up to 12 weeks later to assess stress-related memory retention. Most animals will be killed immediately following the final session to enable blood and tissue(s) to be collected for assessment of plasma stress hormone levels and molecular changes respectively. All trial sessions will be videoed for subsequent assessment using computer assisted analysis software to assess movement, speed, search strategy and success rate.

Scenario 2 (rats or mice): Swimming will be used to induce a highly consistent stress response. The maximum exposure period will be ~5 min for mice or ~6 min for rats which may be repeated only once up to ~12 weeks later to assess stress-related memory retention. Most animals (75% of animals under scenario 2) will be killed following the first session to enable blood and tissue(s) to be collected for assessment of plasma stress hormone levels and molecular changes, respectively. Some animals will be videoed for subsequent behavioural assessment where relevant using computer-assisted analysis software.

Scenario 3 (rats only): A prolonged stress paradigm will be used to identify pathways contributing to stress resilience. Rats will experience an acute stressor up to twice daily for up to three weeks, based on initial experiments to determine the most refined protocol to

deliver the relevant biological responses. Individual acute stressors include tube restraint (up to 30 min), novel environment (~30 min), modest cage movement via mechanical rocker (~30 min), cage tilt (up to 45° static, for up to 30 min), swimming (~6 minutes at ≥25°C, max 1 session/48 h), repeated scuffing (intermittently over 10 minute period), room temperature fluctuations (20-28°C) or changing cage mates (once per week).

Following prolonged stress, all animals will undergo behavioural profiling based on their response to the open field paradigm (10 min), light dark box (10 min), and elevated plus maze (10 min), one exposure each with at least a 10 min home cage recovery between tests. At the end of the study the animals will be killed. The behavioural data obtained from profiling, in combination with data assessing food/water/saccharin consumption, home cage behaviour and endocrine parameters at death including plasma ACTH and corticosterone, and adrenal and thymus weights will be used to classify the behavioural response of rats as ‘sensitive’ ‘typical’ or ‘resilient’ and allow correlation with associated molecular changes. Profiling sessions will be videoed for subsequent behavioural assessment where relevant using computer-assisted analysis software.

Approx. 25% of all animals used in this license will be home cage controls, experiencing only handling and killing steps. Collection of tissue from these animals and subsequent analysis will provide molecular information on pathways involved in non-stressed, basal development.

What are the expected impacts and/or adverse effects for the animals during your project?

Behavioural paradigms:

Adaptive behavioural/stress paradigms (Morris water maze or water paddling test as refinement, rats only; swimming, rats and mice): These animals will show mild to moderate signs of stress (agitation, vocalisation) when exposed to the paradigm but recovery after removal should be rapid, with restoration of normal eating/drinking/sleeping behaviours within 30-60 min.

Prolonged stress (rats only): animals exposed to prolonged stress paradigms are expected to show mild to moderate signs of stress (agitation, vocalisation, aggression, piloerection, weight loss, excessive grooming, shaking) while exposed to the stressors. Signs of stress are expected to remain in some individuals and get more pronounced and remain for longer while they are on the prolonged stress paradigm. Once completed, a proportion of rats are expected to show no overt clinical signs but some non-clinical changes in appetite, sleep patterns, behaviour, until death (up to 12 weeks later), whilst others are expected to resume normal eating/drinking/sleeping behaviours rapidly (24h-1 week) within that time.

Behavioural profiling (rats and mice): (open field, light dark box and elevated plus maze and its derivatives, sucrose (saccharin) preference test): some animals exposed to behavioural profiling are expected to display mild stress responses (vocalisations,

agitation, hiding) during the tests. Once completed, animals should resume normal eating/drinking/sleeping behaviours within 30 min.

Surgical implantation of cannulae/minipump (rats only):

Animals are expected to continue to behave normally throughout the study, with the possible exception of the immediate few hours following recovery from surgery. There is a risk that some animals may experience minor skin irritation at the site of cannula/minipump implantation, characterised by scratching around the implant. If this occurs, it will be controlled by cleaning and the topical application of cream as advised by the NVS.

Substance administration reaction:

There is a risk that some animals may react adversely to neuromodulatory substances following administration resulting in hair loss (2-3 days after treatment) and transient weight loss (<10 % during treatment and for 1-2 days post-treatment). All animals are expected to begin to regain weight within 48h of treatment ends.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- 35% of animals (rats or mice) are expected to experience mild suffering.
- 65% of animals (rats or mice) are expected to experience moderate suffering.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Stress is an active process that can only be experienced by an intact living animal. Consequently, it is not possible to study the physiological/behavioural responses to stress in anything other than a living animal. Likewise, brain development is a process influenced by so many factors, understanding the molecular complexities involved can only come from studies on intact, freely-behaving animals.

Which non-animal alternatives did you consider for use in this project?

Computer simulation programmes. Cell culture lines. Organoids. Human stress studies.

Why were they not suitable?

Computer simulation programmes are not currently available and cannot be developed at present due to the lack of basic information regarding this highly complex area of science, especially for females. Cell culture and organoids will be used to complement findings but the unphysiological and simplistic nature of such in vitro cultures makes comprehensive studies of brain development pathways and behavioural responses impossible. Furthermore, organoids may come across as ‘organ-like’ but their inter-neuronal connections do not reflect the physiology and neuroanatomy of a living brain. Human studies will be used to support our findings where possible but the studies of detailed molecular pathways in humans is not possible.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of rats required over the 5-year period of the license has been calculated using data, obtained from our previous studies, to determine the groups size needed to achieve a statistically valid result. We estimate that 55, provisionally designed experiments, will be needed to meet the study aim. Each experiment will require 2-8 experimental groups comprising of 6-9 rats or 8-24 mice, depending on the outcome being assessed. For studies into the effects of prolonged stress, a group size of 40 animals will be needed, due to the variability in response to such stress, with 1-2 experimental groups per study. Such studies will be undertaken to investigate stress-sensitive and stress-resistant phenotypes. The estimated number includes 400 rats and 80 mice for pilot and contingency experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have worked hard over the years to optimise our experimental design and in house tissue analysis methods to minimise the number of animals needed to obtain the required data.

We have used the NC3R's Experimental Design Assistant application to confirm the validity of our experimental design and have incorporated female animals into our studies to reduce the number of animals that have to be bred to meet our experimental needs.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Due to our experience in this area of brain development and stress research, we have amassed a significant amount of control data which can be used to optimise experiments and avoid repetition thereby minimising animal numbers. In cases where the experiment outcome is unknown (e.g. when evaluating a new RNA or protein) pilot experiments will be conducted initially to determine the effect sizes so that the required group sizes can be calculated.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animals: The studies will use predominantly wildtype rat strains, however, to confirm the involvement of a particular gene(s) or protein(s) in brain development or stress responses, genetically modified animals (generally mice) with either deletion or over-expression of the gene/protein of interest will be used.

Stress models to be used under this license have been carefully considered and selected to minimise the total number of animals required, the number of exposures, the duration of the stress exposure and the severity of the stress experience.

For experiments designed to investigate differences in individual behavioural responses to stress, the Morris water maze will be used (rats only). This paradigm allows us to assess differences in short-term adaptive responses to stress and the impact of these differences for cognition (stress-induced learning). Existing refinements control water temperature and expediate drying time to prevent the risk of hypothermia. Furthermore, under this licence we will assess the water paddle maze as a more refined method to meet our scientific objectives. If suitable this method will negate the need for rats to swim thus reducing the psychological and physical adversity of this paradigm. Active stress coping will be assessed in rats trained with the platform present. A swim control group of rats will be exposed to the maze for matched periods of time (cf. trained group) but without a platform

present to distinguish the stress experience from the active coping component of the behavioural response.

For experiments in which the main aim is to induce an adaptive behavioural response to stress, swimming of rats and mice will be used because it provides a highly consistent result in both species and only requires a maximum of two exposures, thereby minimising both the number of animals needed and the number of stressful experiences required. As such it is currently the most refined method to generate the required behavioural readout of stress adaptation. Again, refinements to water temperature prevents the risk of developing hypothermia. For mice, a higher water temperature will be assessed as a potential refinement given mice lose more body heat and find water more aversive (cf. rats). Rats will be swum for a maximum of 6 min and mice for a maximum of 5 min to reduce the duration of the stress cf. earlier studies A heat mat will be provided under the cage during the recovery period to mitigate any cooling effects in mice. Following an initial swim stress, animals may be retested once up to 12 weeks later to enable assessment of the adaptive behavioural response this stress evokes.

To study the effect of prolonged stress (rats only), an established model of unpredictable repeated stress exposure will be used which has been effective at evoking chronic stress development. The stressors involved in these paradigms are not painful but, due to their un-predictable and regular nature, induce pathological symptoms by overwhelming the coping process in vulnerable individuals. This model reflects the chronic stress condition often seen in humans after exposure to multiple high- impact challenges over a period of time (e.g. exams, job difficulties, court cases etc.). A systematic review of chronic stress protocols that resulted in the most reliable outcomes have informed the duration of our protocol (21 days) and choice of stressors (33% stressors at moderate intensity).

Stressors to be used include restraint (moderate), swimming (moderate), novel environment exposure (mild), cage shake (mild), cage tilt (mild), changes of cage mates (mild), room temperature fluctuations (mild) and repeated scruffing (mild). Initial studies will be undertaken to determine the most refined protocol to use, starting at the minimal level for all stressors, applied at a frequency of 2 stressor per day. If biological objectives are achieved the frequency of stress will be reduced to 1 moderate or 2 mild stressor per day or finally 1 stressor per day. If the biological objectives of the study are not achieved, the level at which the stressors are applied will be increased incrementally as indicated above for each stressor until biologically relevant outcomes are observed or the limits on the protocol have been reached.

Steps will be taken to avoid any unnecessary stress that might be caused as a result of combining similar stressors (e.g. minimising swimming exposure to max. 1/48h; 4 /week). Animals will experience up to two different stress exposures per day, unpredictable in terms of time of day and sequence, over 21 days. This is a refinement against protocols spanning 6-12 weeks in the literature and results in more reliable outcomes than longer time periods. Following prolonged stress the animals may be assessed in a number of

behavioural profiling tests such as the open field, light dark box and elevated plus maze and its derivatives, sucrose (saccharin) preference test as well as assessing food/water consumption and home cage behaviour as measures of anxiety. These profiling tests will allow categorisation of the animals regarding their response to the prolonged stress procedure and, as such, provides more appropriate measure of individual responses than the traditional comparison to non- stressed controls. None of the profiling tests used are expected to cause more than mild stress or result in any long-term harm. Limiting the total duration of prolonged stress exposure to a maximum of three weeks combined with the animals spending the majority (>93%) of each day undisturbed in a non-stressed/home cage environment, offers opportunities for relaxation during the prolonged stress protocol.

Why can’t you use animals that are less sentient?

This type of research requires the use of conscious mammalian model systems to best represent the complexities of brain development and the stress response and hence will result in higher levels of translatability to the human situation. Mice are not suitable for most of these studies as, due to their small size, we would need to use many more mice to get the same amount of brain tissue for downstream studies. In addition, rats display more complex behaviours in learning tasks, have a larger genome size and altered chromosome morphology (cf. mice) making them a better species model to represent the human situation than mice. Mice will, however, be used for some molecular studies involving genetically modified mice because this technology is often not sufficiently developed for rats and data generated from such studies in mice regarding the involvement of (a) certain gene(s) can support and refine targets for more complex behavioural assays in rats.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Optimisation experiments have been performed or designed for all stress/behavioural models to refine these techniques. Before training, all animals (including those which will not be trained) are handled for 5-10 days for 2-3 min/animal/day to reduce non-specific stress following best handling guidance.

Animals are group housed and also have access to cage enrichment such as cardboard tubes/wood chews in home cages. The time rats spend exposed to stress will be reduced in all instances where the scientific outcome will not be compromised. In addition the water paddle maze will be assessed as a possible refinement of the Morris water maze.

Refinements to the prolonged stress paradigms include avoiding unnecessary stress that might be caused as a result of combining similar stressors (e.g. minimising swimming exposure to max. once per day; 4 times per week). Animals will also be cleaned out more frequently to prevent the build-up of soiled/wet bedding while exposed to prolonged stress.

Administration of neuromodulatory substance will be performed using the lowest possible dose and least invasive route appropriate to the experimental design. For novel drugs,

dose will be determined in a pilot study using minimal numbers of rats in a stepped manner until an appropriate dose is reached which is pharmacologically active with minimal side effects. All drugs and solutions will be specifically purchased for use in vivo and made up fresh for the specific experiment using sterile, pyrogen-free pipettes, containers and solutions whenever possible. Care will also be taken to use vehicles at an appropriate physiological pH and osmolarity for use in vivo to minimise adverse reactions.

Where possible non-invasive routes of administration will be used and surgery (to implant osmotic pump, intercranial cannula or directly inject into the brain) will be avoided unless it will substantially reduce the required dose, reduce the duration or stress associated with alternative drug administration methods, or is a requirement for a particular experimental design. For more targeted administration of agents to localised brain regions, rats will either be implanted with a cannula, to be used to administer agents post recovery, or substances will be directly infused under general anaesthesia. Behavioural paradigms (if required) will be withheld for a minimum of 5 days after surgery to allow the animal to recover and then only performed once the animal has returned to a steady state (normal behaviour, eating, drinking, gaining weight etc.). All surgery will be performed under aseptic conditions and with appropriate analgesia to relieve peri-operative pain (monitored using the rat grimace scale and other subtle pain-related behavioural features). New experimentors will be closely supervised by appropriately trained and experienced members of the team and only once they have completed documented training and gained sufficient competence will they be able perform these procedures alone.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow all relevant best practice documents that relate to our work which include but are not limited to Guiding Principles for Preparing for and Undertaking Aseptic Surgery (LASA), Guiding Principles for Behavioural Laboratory Animal Science (LASA), handling and restraint (NC3Rs), Welfare assessment (NC3Rs), The provision of water and food for laboratory animals (IAT).

It should be commented, however, that the LASA guidance document on the forced swim test only refers to use of this test for assessing the behavioural effects of antidepressant drugs. We use swimming as a stressor that elicits molecular, cellular and physiological changes that underpin the consolidation of an adaptive behavioural response (i.e. the learned immobility response) which can be assessed in a re-test, usually conducted 24 hours after the initial swim session. This approach, which was first described approx. 40 years ago, is not addressed in the LASA document.

All data generated from using animals will be published in according with the ARRIVE guidelines (NC3Rs).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Members of the group are committed attendees of NC3Rs meetings and updates are regularly provided by the NIO and our NC3Rs regional programme manager.

116. Functional Consequences of Metals and Mutations On P53 Function

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

p53, chemoresistance, mutations, metals, cancer

Animal types	Life stages
Mice	adult, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to understand how structural changes in the protein p53 change its function. We would like to discover new therapeutics that target this structurally changed p53. Structural changes can be the result of genetic changes in the DNA (mutations) or the exposure to elevated metal levels.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

p53 is the most important tumour suppressor protein, protecting us against the formation of tumours. p53 is mutated in more than half of all cancer patients, often causing the formation of a changed p53 protein (mutant p53) or a complete loss of p53. Interestingly, in cancers, loss of p53 is associated with a milder cancer phenotype than the presence of a mutant p53 protein. Although researchers have studied p53 function for 40 years, we are still not routinely using p53 status in the clinic. Part of the problem is that p53 can be mutated at various places generating up to 10.000 different mutant p53 proteins. We do not know all the functions of these mutant proteins and researchers have not been able to link specific mutant p53 proteins to particular types of cancers or the severity of cancers. More importantly, given that, mutant p53 and normal p53 can reside in various structural states in which they can function differently; we believe that, looking at the different mutations in p53 is not sufficient to associate DNA changes to what is happening in patients.

Therefore, in this project we aim to understand the different functions associated with structural changes in p53 and to detect its structural states in vivo either directly or indirectly. We will then aim to correlate these functional states to patients' characteristics. So far, we have identified new functions of mutant p53 in cannibalising neighbouring cells and in activating certain cellular signalling pathways; the understanding of which will enable us to learn more on mutant p53 function and the differences between p53 mutants. This, ultimately will help us to develop mutant p53 targeting therapeutics.

Alongside, we also believe metals and hypoxia can impact on the structure of p53 and therefore its functional states. Our preliminary data suggest that, with a certain treatment we can restore normal p53 function in some p53 mutants, allowing them to act as a tumour suppressor. In summary, our knowledge and ongoing studies on mutant p53 and the structural changes in p53 will be integral to increase our knowledge and be able to provide therapeutics that can target mutant p53s in cancer, which would then greatly benefit cancer patients.

What outputs do you think you will see at the end of this project?

Publication, patents, new therapeutics. This project will result in several publications and we will aim to develop therapies that could work in the patient setting. A patent application for a new drug strategy has been submitted to the European patent office.

Who or what will benefit from these outputs, and how?

The University and members of my lab, including myself, will benefit from publications in the short term.

In the long run, we aim to address whether certain endogenous metals are carcinogens, which will be valuable information for organisations such as the world health organisation. Information on this is currently limited.

In 5-10 years we aim to test therapies that restore p53 as therapeutic strategy in cancer patients that are affected by metal accumulation in cancers.

How will you look to maximise the outputs of this work?

We will be collaborating with various researchers and industry to develop this project. We will present our work at international conferences and publish in world class journals.

Together with an industrial partner we aim to develop the strategies that restore p53 function into a viable approach to treat cancer patients in the future for which we aim to distribute our knowledge to oncologists.

Species and numbers of animals expected to be used

- Mice: 900

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will be using a combination of immune compromised mice and normal mice. The advantage of the immune compromised mice is that we can determine how the different p53 mutant human cells are behaving, without causing an immune response in these mice. We will administer human cancer cells to mice and determine how the tumour grows in the presence of changing conditions such as chemotherapeutics and various other therapeutics as well as metal exposure.

Normal mice will be used to determine whether metals are able to form tumours and whether that is p53 dependent. To get a full picture of how metals act on p53, we will also use normal mice to study other roles of p53 in cell movement. p53 has been shown to play a role in wound healing and embryo development. It could be that p53 is regulating the movement of cells necessary in these processes, similar to how mutant versions of p53 play a role in cancer cell movement. For this research we will use normal young adult mice as well as embryos to determine the role of metals in p53 function. We have chosen young adults so that we can compare them later with p53 KO mice (through collaboration). These mice can only be used in early life to study such processes as otherwise they are likely to

develop cancer. If we are to target unfolding of p53 it will be important to understand its function in other processes that we could otherwise unknowingly compromise.

Typically, what will be done to an animal used in your project? immune compromised mice and normal mice- tumour formation:

Metal accumulation typically takes time and might therefore be administered as a few injections (typically once a week for 3-6 weeks) before cell injections. Alternatively, when looking at lung cancers, mice inhale metals under anaesthetic 2 times a week for the duration of 6 weeks maximally

Typically, we will inject cells under the skin, in organs or in the blood stream. These experiments typically are done under anaesthesia and it will typically take 4-12 weeks for tumours to form. Tumour formation can be felt or measured with a device called calliper or imaging

After tumours are formed, we will administer chemotherapeutics or other therapeutics. Chemotherapeutics and other therapeutics will typically be administered after tumours are formed and are typically administered once a week or twice a week intraperitoneally until the tumour reach a maximum size.

Once tumours reach a maximum size or a certain amount of predetermined time since injection has passed, mice will be humanely euthanised and organs/ tumours harvested for histology and other readouts.

We will also initiate tumour formation in the lung using carcinogens such as benzopyrene (found in cigarette smoke) and allow tumours to form in metal-loaded versus normal mice. If tumours are formed we will preferentially test our therapeutics in this more patient-like model. Tumours might arise quite late though, up to 9 months after exposure to benzopyrene. We will however stop experiments after 1 year as it is unlikely that at that point still tumours will develop from our initial treatment.

Normal mice - wound healing:

optional: Metals/ therapeutics will be administered prior to wounding

Wounds will be made under general anaesthetic at the back of the mice (collaboration with a colleague experienced in this area)

optional: Metals/ therapeutics will be administered locally

Mice will be euthanised at different time intervals after wounding typically 8 hrs, 16 hrs, 3 days, 8 days and 14 days after wounding to study p53 expression (folding), gene expression and metal levels throughout wound healing.

Normal mice- embryogenesis:

Metals will be administered prior to fertilisation

Mice will be allowed to mate and at certain times after fertilisation- typically 8-17 days, pregnant females will be euthanised and embryos investigated for metal levels and p53 folding/ gene expression

What are the expected impacts and/or adverse effects for the animals during your project?

It is expected that mice will develop tumours. Subcutaneous tumours rarely cause any visible harm to animals. On occasion, animals might develop an infection around the injection side, which we will try to avoid using aseptic techniques for injecting these cells. Other problems might occur due to necrosis of the tumour tissue (tissue dying in the centre of the tumour), although we have not observed this with the cell lines we have used so far.

Injecting tumour cells in the tail of a mouse can result in lung tumours. At first sign of laboured breathing or weight loss, mice will be humanely euthanised. Because of previous experience we should know at what time interval we are best to cull animals and prevent any severe effects. These experiments will be classed as moderate severity, but in reality, less than 10% will experience moderate effects. The majority will be mild

Inhalation of metals or carcinogens can cause acute breathing problems. These compounds are given under anaesthesia so if such a problem arises, mice will be culled immediately. Acute breathing problems in conscious mice are considered severe, but as we administer this under anaesthesia and we will monitor for the first 30 seconds we will be able to cull any animals before this severity level is reached if needed. Less than 5% will have breathing problems

Wound healing is considered moderate and mice will be given analgesia for the duration of the wound closure. Infection is a possibility and is typically seen in less than 1% of animals. Using sterile techniques will avoid this

For euthanasia the most humane and NC3R approved methods will be implemented to ensure the least suffering of mice. We will use a combination of the approved schedule 1 methods cervical dislocation and terminal anaesthesia, dependent on the material we will need to prove our hypotheses.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Animal	minor	moderate	severe
SCID gamma subcutaneous xenograft	90	10	
SCID gamma tail vain injections	80	20	
SCID gamma tail vain injection/ metal inhalation	20	80	
Bl6/ p53 KO tumour formation lung tumours metal inhalation	20	80	
Bl6/ p53 KO wounding		100	
Bl6/ p53 KO embryos	100		

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In this project we are investigating how to treat tumours with mutations in p53 using new therapeutics. In addition, we want to characterise how p53 responds to metals. We will make use of various cellular model systems, but these are currently not sufficient to predict behaviour of therapeutics in a whole organism. In addition, we are looking at complex processes that consist of multiple cell types for which no adequate cell models exist. Invasion and metastasis are very much dependent on the environment and cellular model systems can only encompass small aspects of these processes. For mutant p53's function in engulfing other live cells, our key question to address, is whether the phenotype we see in tissue culture is reminiscent of certain cellular structures that can be observed in tumours of animals and humans. The best way to prove this is to study this process in clearly defined pieces of tumours, established from cell lines with different fluorescent colours. Similarly, wound healing and embryogenesis involves a variety of different cells and signals that cannot yet be studied to the full extent in tissue culture models. Embryogenesis will mostly be studied using lower eukaryotes, but these animals do not

express all isoforms or family members of p53, which will limit the information we will get from them.

Which non-animal alternatives did you consider for use in this project?

For tumour cell spreading, we will be using animal free products such as hydrogel and growth factors derived from bacteria to create 3D environments and study cell movement. For wound healing we will work together with a colleague who has established a 3D model of wound healing. For cell engulfment we will use 3D products such as mentioned before and we will also suspend the cells in such a way that a more 3D-like condition is created. All these model systems will be used to better predict how cells will behave in animal models and will limit our use of animals considerably.

Why were they not suitable?

Wound healing, cancer cell spreading and embryo devlopment are processes that involve many different cells and are therefore not easily modelled in the lab. If you give compounds in a dish, these can be immediately taken up by the cells, but in an organism they often are degraded or do not reach the site where you want them to act. Compounds might therefore not be active in the actual tumour. In order to convince clinicians to test metal reduction strategies in humans, we need to provide proof-of- prinicple in a whole organism that is more similar to a human, such as a mouse.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have calculated for each step in our licence how many mice we maximally would need to be able to prove that our hypotheses are correct. This was based on an online tool called EDA available on the NC3R website On some occasions these include pilot experiments to make sure that if we inject cell lines that we never worked with in animals before. This is tho make sure that these do not give rise to adverse reactions in the animals. In addition, we will be changing p53 status and we will need to know to what extent such changes potentially already cause differences when you establish the tumours.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

As we already have preliminary data from previous animal work, we used these experiments to guess how many mice would be needed to make sure that our findings will be robust. We have used the EDA from the NC3R website to calculate the minimum number of animals one would need to make sure that the effect you see is a true effect. For this we used our own data, but also carefully studied various literature articles by others to determine carefully how big of an effect we expect to see.

We have carefully determined which cell lines would be most suitable for this project and we have decreased animal numbers as much as possible by combining experiments so that controls could be used for multiple readouts, limiting numbers of mice used in multiple experiments. In addition, for some projects we will need to grow tumours to have ex vivo material available for studying cell engulfment.

We aim to combine these experiments with other parts of the projects/ experiments in which we simply want to see how fast tumours are growing. We will aim to work together with others and reduce mouse numbers even further if possibilities arise in the future.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As mentioned above, we will be doing pilot experiments with small numbers of mice to better predict estimated effects and with that to better determine how many mice would be needed for any large experiments to make sure that the effects we will detect will be deemed true. We will work together with other researcher in the field of p53 to minimise mouse numbers and will aim to share tissues when possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Non-SCID gamma mice that are immune deficient to allow implantation of human tumour cells to avoid that these cells are rejected by the animals. We will study tumour growth in various places within these animals. Suffering will be prevented by monitoring tumour growth by palpation when possible. If this is not possible mice will be monitored regularly to avoid any unnecessary suffering by scoring weight and general appearance

Bl6 normal mice to study wound healing and embryogenesis will be used to be able to compare to p53 KO (lost p53) mice that have a Bl6 background. Others have used these mice for studying these processes which makes it easier to compare studies.

Why can’t you use animals that are less sentient?

If we can, we will use Drosophila (fly) and C.elegans (worm) to study embryo development. Problems with these species are the exposure route of metals might be very different compared to how humans or mice are exposed to metals. These animals also have considerable differences in the p53 protein, so this p53 might not be fully similar to human or mouse p53 (these are more similar) in their response to metals.

For tumour work, the mouse represents a model that is most comparable to the human situation. Drosophila has been used to study tumour growth, but cancer spreading cannot be monitored in this model. We will perform a few pilot experiments to see if any of our work on testing compounds can be done in Drosophila to reduce animal models in subsequent experiments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Once tumours have established, we will increase the monitoring frequency of the animals to make sure no unnecessary harm will be inflicted. Similarly, when wounding animals, we will increase monitoring frequency. We will also apply pain management where needed.

In our institution, there is a single use needle policy and good a-septic technique that will help in refinement

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidelines described in the following paper for oncology models: https://www.nature.com/articles/6605642

For general guidelines we will refer to the NC3R website and LASA guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am in the grant panel of the NC3R, which will ensure that I will be aware of any of the latest updates in all animal work. I am actively participating in evaluating new model systems for replacement of animals and I will make sure I will use these if I can think of how they best fit in my work.

117. Developmental Effects of Schizophrenia Risk Variants on Gene Expression

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Schizophrenia, Synapse, Transcriptome, Grin2a, Trio

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify patterns of gene activity associated with schizophrenia genetic risk across different stages of brain development and different cell types, focusing on the synapse.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Schizophrenia is a chronic and severely impairing disorder. The development of much-needed new treatments has stalled due to a lack of understanding of the underlying biology. Recent discoveries show that genetic effects may preferentially impact sites where signals transmit between nerves (neuronal cell synapses), but we do not yet know which specific components of a synapse are most vulnerable and at which timepoints. New breakthroughs in genetics research have made it feasible to model the genetic effects and investigate synaptic vulnerability relevant to schizophrenia and related disorders. This information is important for facilitating the design new treatments.

What outputs do you think you will see at the end of this project?

This research will expose subsets of proteins important for development of synapses that warrant further investigation in studies of schizophrenia or may be amenable to manipulation through drugs. Importantly, the results will highlight the developmental stage and cell type in which the generation of these proteins is most relevant to schizophrenia, thereby providing key information on the context in which future research should focus.

Large sequencing datasets describing molecular biology in synapses at multiple stages of development will be generated and made freely accessible as a lasting resource for further interrogation.

Who or what will benefit from these outputs, and how?

Resulting datasets would likely be taken forward by cell biologists or pharmacologists interested in generating new disease models and understanding the mechanisms underlying psychiatric disorders. Datasets will be released upon first publication.

Cell-specific sequencing datasets from mice carrying genetic variants will benefit researchers interrogating the function of synaptic genes and their involvement in diseases or disorders beyond schizophrenia, including epilepsy and developmental delay.

Novel informatics methods written for investigating relationships between genetic association with schizophrenia and patterns of gene expression will be of benefit to studies of other related disorders. All programming code developed for this research will be made available for such uses through GitHub repositories and regularly updated throughout the period of the project.

How will you look to maximise the outputs of this work?

I will prioritise transparency and open science in the communication of my primary research outputs. I will disseminate new findings through major international conferences, such as the World Congress of Psychiatric Genetics, as well as internal and external talks, including contributions to meetings of the Schizophrenia working group of Psychiatric Genomics Consortium. These important interactions will be used to acquire feedback and seek new collaborations to maximise the impact of my research. Ahead of peer-review, I will ensure early release of all scientific manuscripts through pre-print servers.

Research articles will be targeted for publication in reputable journals with open access and transparent peer-review. Electronic copies will be deposited immediately in PubMed Central with links to raw data made available through public data repositories.

Species and numbers of animals expected to be used

- Mice: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice at embryonic, early postnatal, adolescent, and adult stages of development. Schizophrenia and related disorders are widely considered to have developmental origins. Therefore, to understand how genetic mutations lead to relevant pathology, it is informative to study the animal models both as their brain develops and during adulthood.

The mouse provides an appropriate model of human synapse development due to the similarity in synapse complexity, composition and function. The genes being studied are similar between human and mouse, and therefore changes observed in the mouse can be assumed to have functional implications relevant to human physiology.

Typically, what will be done to an animal used in your project?

We will breed colonies of mice of which some will have targeted genetic mutations. Groups of these mice will be allowed to develop to a range of ages before being culled humanely for tissue collection.

What are the expected impacts and/or adverse effects for the animals during your project?

A subset of mice with genetic mutations are likely to exhibit minor immune or metabolic deficiencies throughout life, with the chance of additional minor behavioural abnormalities from 10 weeks of age in the form of reduced motor coordination and increased anxiety.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice carrying genetic modifications (approximately 50%) are expected to be subjected to a maximum of moderate severity of harm, with most experiencing mild or sub-threshold

harm. The remaining mice carrying wildtype copies of the genes of interest are expected to experience sub-threshold severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project aims to answer important questions concerning brain development in the context of genetic mutations relevant to neuropsychiatric disorders. In particular, TRIO and GRIN2A genetic mutations, which considerably increase schizophrenia risk in humans, are very rare in the population. Therefore, it is not feasible to acquire post-mortem samples of developing human brain tissue affected by the mutations. Instead, the genetic variant must be induced in a model organism. In order to capture biological systems at critical developmental stages relevant to the human disorder, samples from brain tissue must be studied as they would be found in a living organism. This is particularly important for the study of synapses, whose development and structure are finely tuned by environmental stimuli. Hence, for the fulfilment of the aims, the genetic variants will be modelled in mice.

Which non-animal alternatives did you consider for use in this project?

Since targeted genetic mutations can be made in stem cells, the use of a cell culture approach has been considered for modelling variants in genes such as TRIO and GRIN2A.

Why were they not suitable?

At present, neither normal brain development nor synaptic activity is sufficiently modelled in cell culture for answering our research question, which demands comparisons across developmental stages corresponding to critical time periods for psychopathology in humans. However, as progress in this area accelerates, stem cells or organoids may become a valid alternative in future.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimates are based on an average litter size of 6 and accounting for variance in genotype and sex distributions. The total number of animals corresponds to that required to achieve the objectives of the research with a sufficient level of confidence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Colony size planning has been prepared using Jackson Laboratory resources. Sample sizes were based on variance estimates provided by previous research using similar techniques, and statistical modelling methods provided by collaborators with relevant experience.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Firstly, the amount of available RNA per sample will be maximised by crossing mouse lines with a RiboTag mouse strain, or similar, which allows for optimal RNA extraction. Brain tissue from each mouse will be split for use in two downstream experiments instead of generating more animals. This strategy will further contribute by increasing the validity of comparisons between the experiments. Our breeding plan ensures that all offspring are usable.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mouse models of genetic risk factors contributing to Schizophrenia, such as Trio and Grin2a genetic loss of function and cell targeting models such as Ribotag. The models used may cause minor behavioural, immune or metabolic abnormalities. Selective breeding of each strain will ensure any mutation is only present in one copy of the gene (heterozygous), thereby reducing the severity of adverse effects.

Why can’t you use animals that are less sentient?

We aim to model aspects of biology associated with psychiatric disorders such as schizophrenia, a complex psychopathology only found in humans. Therefore, the animal

should share sufficient biology with humans such that our findings can translate to human health.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Where possible, we will use heterozygous genetic alterations rather than homozygous variants to reduce the occurrence of adverse effects. Newer animal models for which the effects of the genetic mutation are less well known will be monitored more closely, particularly in new litters. If concerns are raised, animals will either be culled, or permission sought from the Home Office to maintain the line and an amendment made to the PPL to include the relevant humane end-points for the strain.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3Rs organisation provides resources for refining animal research, including those for generating and breeding genetically altered mice. Welfare assessments will be conducted in line with published guidance (Wells DJ, et al 2006, Assessing the welfare of genetically altered mice, Laboratory Animals) and more recent guidance signposted by the RSPCA (Hawkins P, et al 2014, Progress in assessing animal welfare in relation to new legislation: Opportunities for behavioural researchers, Journal of Neuroscience Methods). Long-term refinement will be optimised by following Norecopa PREPARE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As a team, we will stay informed of 3Rs updates provided by Norecopa and NC3Rs through online media. Method developments and alternatives to animal use will be tracked through reading journal publications and pre-print articles, paralleled by relevant conference attendance. Throughout the project I will take advice from the NVS and NACWO on the refinement of current methods and implementation of new ones. I will maintain an interaction with the local Animal Welfare and Ethical Review Body to remain informed of local 3Rs updates. Regular team meetings dedicated to 3Rs advances will take place to distribute updates and provide training so that new actions can be implemented.

118. Cartilage Repair and Replacement

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Orthopaedic, Knee, Cartilage, Biomaterials, Therapy

Animal types	Life stages
Sheep	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To determine the effectiveness and durability of novel orthopaedic replacement therapies. As well as to assess the best attachment methods for this novel technology.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Osteoarthritis is a crippling disease caused by damage to cartilage and the underlying bone in the body's joints. Arthritis Research UK have estimated that osteoarthritis will cost the NHS £118.2bn between 2017 and 2027. The only widely accepted surgical treatment for osteoarthritis is replacement of the joint with a large, permanent implant. This requires

major surgery which takes several months to fully recover from and while often successful there are variable outcomes and it is not recommended for younger patients. As osteoarthritis can develop early in a person's life this means many patients cannot currently be treated effectively.

What outputs do you think you will see at the end of this project?

The initial outputs will be information regarding the attachment suitability and device longevity, integration and function. Which will form the basis of longer term studies, in the future, for regulatory approval of the device. With the aim of creating clinically available implants, potentially benefitting thousands of patients, in the UK alone, improving their mobility and, as a result, improving their overall quality of life.

Who or what will benefit from these outputs, and how?

According to the UK National Joint Registry over 117,000 surgical knee procedures were carried out in 2019 in the UK alone with over 4 million knee cartilage lesions discovered by routine arthroscopy in the EU and US each year. Whilst this technology would not necessarily be useful to all of these patients, over 1.1m of the knee cartilage lesions discovered were full thickness with associated osteoarthritis in many cases, and these form the primary target clinical population for this technology.

However, as the technology could potentially also be applied to alleviate similar problems in joints other than the knee, the products developed under this licence could be useful in many times this initial number of patients. As a result, while it is not possible at this stage to provide a meaningful estimate of the total number of people this technology would benefit it is clear that the number is very large and a conservative estimate of 50,000 patients annually in the UK may benefit.

How will you look to maximise the outputs of this work?

As much of the work is for the development of commercial products and is therefore, covered by non- disclosure agreements, it may not be possible to publish data from this study until after the product is in clinical use, due to patent and confidentiality issues. However, it may be possible to share the animal model information independently of the product information and it may also be possible to share/publish the information arising from subsequent human clinical trials/usage.

Species and numbers of animals expected to be used

- Sheep: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The hind leg of a sheep is a proven model for human knee research because the anatomy of the sheep knee is very similar to that of humans. We need to use adult sheep as the animals need to be skeletally mature and also adult sheep body weight is comparable to that of an adult human.

Typically, what will be done to an animal used in your project?

Animals will be brought in and allowed to acclimatise for a few weeks - this helps them to relax in their new surroundings, gets them used to the staff and human contact and enables them to be monitored for general health or other issues. Prior to surgery they will undergo general observations as to their overall health and specifically how they walk. Food will be withheld immediately prior to surgery, as is done with human patients, to prevent possible problems with regurgitation (vomiting). Animals will also be individually housed (but within close proximity and line of sight to the rest of the 'flock') in the 24-48 hours prior to surgery. In our experience, individual housing for a couple of days prior to surgery means it is often less stressful for them when they are individually housed post-operatively.

On the day of surgery the animal will be anaesthetised (including pain relief), blood taken and non- invasive imaging (e.g. x-ray) may be performed on the hind legs. The relevant area will then be exposed and a defect created in the bone, and/or meniscus (a pad of cartilage, between bones, which acts as a shock absorber), to which the new implant/therapy will be applied, to simulate humans undergoing damage repair surgery. The operative site will then be sutured closed and the animal given pain relief, allowed to recover from the anaesthesia and returned to its pen. The animals will be observed closely for the first week, given more pain relief as required, and monitored for general health and mobility for the duration of the study. At several time points throughout the study the animals will be specifically assessed for how well they are able to walk, as well as x-rays taken, and/or other non- invasive images or assessment methods used. Blood samples will also be taken over the course of the study.

Initial studies are planned to be of 3-6 month duration with follow up studies being up to 2 years long to assess long term recovery and implant integration.

Where necessary, animals will be housed singly during the early phases of their post-operative recovery but group housed again as soon as possible. The reason for individual housing is to allow healing to begin and reduces the risk of problems as the sheep has time to recover before re-joining the group.

What are the expected impacts and/or adverse effects for the animals during your project?

Based on previous experience using this model, we would expect to see:

- A slight weight drop (usually less than 10%) after surgery followed by a return to normal weight, within a few weeks. Some of which may be due to clipping of the fleece as part of the surgical access/preparation.

- Some evidence of pain/discomfort related to the original surgical incision and manipulation of the joint, usually controlled with medication and resolving within a few weeks.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sheep – moderate – 100%.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cartilage is a complex tissue which interacts with the surrounding bone and joint tissue and whose growth and maintenance is affected by the mechanical stimulation of joint motion and weight bearing. These conditions are almost impossible to replicate in synthetic models or dead tissue, especially when looking at healing over longer time frames (i.e. months). Also, it is not possible to use smaller species as the size of implant used, the weight applied to it and associated surgery becomes far less comparable to humans.

Which non-animal alternatives did you consider for use in this project?

We could not find any suitable, non-animal alternatives, however, the devices will have undergone in vitro and ex vivo testing, where appropriate, prior to the in vivo phase of the work.

Why were they not suitable?

It is not yet possible to fully simulate an intact biological system such as the knee especially under the conditions that occur during walking and other movement. A living system is necessary to assess fixation strength, integration, healing and remodelling under these conditions.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on several years' experience using this model (over 100 sheep), we found that:

A pilot study, usually of shorter duration (e.g. 1 month) and using fewer animals (e.g. 4 or less)

Followed by longer (e.g. 3, 6, 12 or 24 months), larger studies (less than 10 animals per test group at each time point)

Gives the best results, with the least number of animals used.

It is sometimes necessary to carry out more than one pilot study, depending upon initial results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The knee joint at the end of the femur is made up of two separate, but similar, boney structures called condyles. As we only operate on one of these condyles, it is possible to use the other as a control (within the joint) and the matching condyle on the opposite hind leg can be used as an unoperated knee control.

Non-invasive imaging will also be used, where possible, to allow data collection at various time points, without surgical site disruption or the need to kill animals at each timepoint. Previous studies using identical or near identical surgical techniques have been carried out by the same team (surgeon and pathologist), including just-defect-creation controls, as such, unless we make a major modification to the operative technique, these data can be used as controls, removing the need to have separate controls for each individual study.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The use of dead tissue to refine any changes to surgical technique.

The increased use, where possible, of non-invasive imaging to avoid having to kill animals at multiple time points.

Tissue sharing wherever possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The sheep model is considered the closest mechanical representation of the human knee joint (although there are several acknowledged differences) and the animal weight is similar and should not alter significantly over the course of the study. There should be little discomfort for the animal from the surgical site and any discomfort would more likely arise from the surgical access. As this is a surgical operation on the knee as opposed to an uncontrolled traumatic injury to the knee (which often involves significant damage to multiple parts of the knee joint) this should be more than adequately controlled by standard post-operative pain killers.

Why can’t you use animals that are less sentient?

Due to the size restriction of this technology it is not possible to work on smaller animals nor is it possible to work on animals that are not skeletally mature due to growth/expansion issues.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The use of best surgical practice and adherence to the principles set out in the LASA (Laboratory Animal Science Association) guiding principles document combined with good pre-, intra- and post- operative care and monitoring will minimise unnecessary suffering. The use of non-invasive assessment (e.g. MRI or X-ray), whilst potentially increasing the number of anaesthetics an individual animal has over the course of a study, can significantly increase the amount of information gained per animal and therefore reduce to overall number of animals used. Also, with a degree of animal training and familiarisation and the correct pre-medication (often delivered in food rather than by injection), the stress/suffering to the animal can be minimised - this applies to medication delivery, acclimatisation to single housing, blood sampling and any other events that require interaction with the animal. By combining as many procedures as possible, it should be possible to reduce the number of anaesthetic events each animal undergoes.

Again input/ support from the local NIO, NACWO, NVS and other local animal care staff will greatly help with this.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The Norecopa, NC3Rs and LASA (and similar animal research and welfare) websites

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Review of the current literature (also encompassing changes in veterinary, research and human surgery) and any revisions to the regulatory guidelines along with input from the local Named Information Officer (NIO), Named Animal Care Welfare Officer (NACWO), Named Veterinary Surgeon (NVS) and other local animal care staff. As well as checking the Norecopa, NC3Rs and LASA (and similar animal research and welfare) websites

119. How Does Gene Expression Control by Rna Binding Proteins Enable Germ Cell Development?

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

RNA biology, gene expression control, development, RNA splicing, testis development

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this study is to advance our understanding of how gene expression is controlled in normal testis development and how defects in these normal healthy processes may underpin male infertility. Some of our genes of interest are also expressed in other organs, particularly the brain, so we aim to find out if we can extrapolate our analyses of testis development more broadly to understanding functioning of other parts of the body.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Our research will investigate how RNA binding proteins control gene expression pathways in the testis. The testis is one of the most active sites of ongoing development in adults and includes the need for cells divide and change eventually into sperm. Defects in RNA binding proteins during infertility can lead to infertility. Why this is is not well understood, and this is what we want to find out. Our preliminary work shows that the RNA binding proteins we are investigating are important for male fertility (see Scientific Background section). However, we currently lack important details of which cell types these RNA binding proteins are important for, what targets they regulate and why these are important for male germ germ cell development, and how these RNA binding proteins cooperate with each other in vivo. The testis is difficult to model using in vitro systems, so we need to use animal models. The research that we undertake using genetically modified animals will relate to what might be going wrong in some forms of male infertility. Since many genes expressed in the testis also function in the brain, our research may also help us understand how other complex tissues like the brain work.

These studies investigating the mouse will help us understand what is happening in humans. This project is important as it will enable us to understand more fully how genes are expressed in whole animals - a process that is crucial for normal development.

What outputs do you think you will see at the end of this project?

Expected outputs will be the identification of the genes and processes that are controlled by important splicing regulators during male germ cell development and revealing the developmental consequences when this splicing control is disrupted. These splicing regulators will include Tra2beta and RBMXL2.

This will lead to new knowledge of basic biological processes that are required for normal male fertility. Our data will help reveal more broadly how gene expression is controlled during animal development. The outputs of this information will be research papers, animal models, and RNAseq datasets.

Who or what will benefit from these outputs, and how?

Who will benefit? Scientists and clinicians interested in male infertility (which affects about 10% of couples). More generally our proposed work will benefit scientists and clinicians interested in how gene expression pathways contribute to normal and abnormal development, the development of new animal models, experimental approaches or identification of new pathways which could be targeted to yield therapeutic benefit.

How will they benefit? Male infertility is often unexplained, but often is associated with defects in RNA binding proteins. What these RNA binding proteins are controlling is often

unknown. A major benefit of our proposed work is it will identify genes and pathways that are controlled by RNA binding proteins necessary to maintain normal male fertility – providing knowledge that will help to understand what is going wrong in male infertility. The identification of genes and pathways important in male infertility will also benefit scientists interested in other tissues that are much more difficult to analyse like the brain. For example, loss of Tra2beta prevents brain development, making it really difficult to identify downstream target genes in the brain as the relevant cell types are gone. Using testis as a model we can successfully identify target genes since loss of the testis does not compromise viability, and our published data suggests that these same target genes will also be important for brain development and function. Scientists and medics will benefit by reading our research papers which will share our results, conclusions and ideas for the future; by open access to the global datasets we generate (with links to these datasets being provided in our research papers), and by free access to our mouse models.

Timeframe. The impact of these outputs should be in the 2-8 years, as we publish papers during and after the completion of research projects.

How will you look to maximise the outputs of this work?

We will present the results at scientific meetings, and through publication in peer reviewed journals, so that other scientists become aware of the outputs of our work. We will aim to publish papers in an "open-access" format, where they are available without needing a subscription, and post also onto web servers that contain early versions of manuscripts (called "pre-prints" - again these are easily available to anyone with access to the internet). We will also use social media (e.g. twitter) to provide updates on our work to a wider audience (including summarising key papers and presentations). We will maximise outputs by collaborating with other groups within Newcastle University who are experts in neurology, behaviour, development and fertility, and can in turn provide an interface with other groups of scientists and medics. We will also collaborate nationally and internationally with other scientists who are interested in how these gene expression pathways work in both normal health and become compromised in male infertility and other diseases.

Species and numbers of animals expected to be used

- Mice: 8000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Types of animals: We will do our experiments in mice. This will enable genetic approaches to identify physiological targets in a system that is a close model for understanding

humans. We need to use animals since the complex developmental pathways and tissues we want to investigate are difficult to model in vitro, but important for normal health. This particularly applies to male germ cell development that occurs in the testis but is very difficult to model in vitro using cultured cells.

Choices of life stages: Different steps in testis development occur at specific ages, so we want to study all ages of mice. This will include adults, since spermatogenesis is highly active in adults (e.g. the human testis produces around 45 million sperm/day) but important stages in sperm development are present at all ages. We will also use genetically altered mice to investigate how these same nuclear RNA binding proteins contribute to other aspects of development and tissue function including the brain, ovary and other tissues. This will require using male and female mice of different ages.

Typically, what will be done to an animal used in your project?

Mice with genetic alterations will be bred and used for analysis. Mouse tissues will be harvested and used for ex vivo analysis. We will isolate tissue to examine patterns of gene expression, protein expression and tissue architecture. Most mice will be terminated using a schedule 1 procedure. In some cases where it is not appropriate to do this (e.g. when tissue architecture is important) mice will be terminated by other methods (such as perfusion of fixative under terminal anaesthesia). In some cases we might need to get assistance for specialised techniques like electrophysiology (carried out on ex vivo tissue) and behaviour. In this case, some animals will be bred in this license for experiments that are covered by separate licenses held by other investigators (for example, in previous research we identified splicing defects in genes important for neurological function, so we supplied our mice for other investigators expert in behavioural analysis and neurophysiology).

What are the expected impacts and/or adverse effects for the animals during your project?

Changes in gene function within the testis may cause male infertility and a reduction in testis size, but not result in pain or discomfort. Some genes are only important in the testis - when we modify these genes most of the impacts/adverse effects will be minor in terms of animal suffering. Other genes that are important in the testis may also be more widely expressed in the body. In this latter case we can still direct genetic manipulations within the testis and other tissues using mice that express special proteins called "Cre recombinases". In some cases these "Cre recombinases" can be active outside the testis at some level, and in this case their use can cause effects in other cells and tissues that are not expected. To assess this, mice will be visually monitored by us and our animal facility, and if there are any additional unexpected defects we will take appropriate action. For example, we have noticed that use of Hspa2-Cre mice to delete the Tra2beta gene causes unexpected effects in tooth development that can affect eating, and consequently

we use a soaked diet and teeth trimming. We will also where possible use young (neonatal) mice to avoid any complications that might arise later on

in development. In the case of any sign of discomfort to the mice we will consult with the experts in the animal facility and the NVS/NACWO.

We anticipate that some of the gene expression pathways that we identify will be relevant outside of the testis as well. For this reason, some of our experiments will address gene expression in organs other than the testis - and particularly the brain. These experiments could potentially cause adverse effects, so mice will be carefully monitored to detect these. These genetic alterations may also cause changes that are biologically important but not associated with suffering or discomfort. For example, one of our mouse models that inactivates the Slm2 gene affects gene expression within the brain causing defects in hippocampal function, but these defects are only detectable using electrophysiology and detailed behavioural analyses, and do not cause any obvious animal suffering. Ourselves and the animal facility staff will visually monitor the mice produced in this project, and in the case of any sign of discomfort to the mice we will consult with the NVS/NACWO.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Only mice will be used in this program of work. We estimate that most (>90%) of animals used will in procedures that are mild including breeding mice to maintain a colony, and for obtaining tissue for ex vivo analysis. The remaining ~10% of mice will be used in procedures that are moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our main focus is how RNA binding proteins control germ cell development. Spermatogenesis cannot be recapitulated outside an animal, so to analyse gene function within developing germ cells we need to use testes to get the cell types we want to analyse.Many of the targets genes we are investigating are only expressed in particular cell types within the testis, or during development, so are impossible to study these genes without using animal models. Experiments on mice further enables us to monitor outcomes

of changing gene function on whole animal biology (e.g. on male fertility and germ cell development). Thus we can actually detect the phenotypic effect of removing specific RNA binding proteins on whole animal biology. Some genes expressed in the testis are also expressed in the brain. In some cases we will also monitor effects of RNA binding proteins on brain gene expression. Brain cells operate in networks involving precise interactions that cannot be easily modelled properly outside of tissue.

Which non-animal alternatives did you consider for use in this project?

We have considered cultured cells.

Why were they not suitable?

Tissue culture cells can be used for some experiments. Once we have identified target genes that are controlled by our RNA binding proteins we can then further investigate the mechanisms by which these operate using transfected cells. However, experiments that purely rely on tissue culture cells can often provide misleading data. The reason for this is that cells grown in tissue culture are usually cancer cells, so quite unlike normal healthy tissue. Some aspects of gene expression we are interested in do not happen properly in tissue culture cells - which are often from different bits of the body than the testis or brain. For example some Tra2beta target exons like NASP-T are only expressed in the testis and embryonic development, and the RNA binding protein SLM2 is only expressed at high levels in the brain and the testis. To use cultured cells we often have to "over-express" particular genes we are interested in, which can in turn change how these genes operate. So we can use tissue culture cells, but it is critical to use actual tissue as well. Tissue culture cells are also usually homogeneous cultures grown either in suspension or on plates, so lack the complexity that is important for modelling tissue function (tissues contain multiple cell types that interact). Cultured cells are also less useful for measuring the outcomes when specific gene expression pathways are not operating properly -for example infertility or behaviour. Despite these drawbacks, tissue culture cells can be very useful for testing predictions based on animal models. We will routinely use cells in culture where they are useful to dissect the biological mechanisms of gene expression.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals estimated is based on the programme of work planned and existing experience of the minimal number of animals needed in each experimental group

to provide statistically robust outcomes. Much of our work involves analysis of gene expression control in spermatogenesis. We are using small numbers of litters, so often need slightly more litters than predicted because of imbalances from expected ratios between males and females and different genotypes. A major output of our studies will be to produce ex vivo tissue for RNAseq analysis, where 4 biological replicates of experimental and control mice provide a good compromise between (1) enabling sufficient depth of sequencing for each replicate in a typical NextSeq 500 run (that might contain several experiments in parallel); and (2) enabling us to distinguish between variability caused by our experiment and biological variability. It is also consistent with the most up to date analysis packages for RNA-seq using multiple replicates.

We have also consulted with the AWERB statistician in estimating these numbers.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Group sizes are constantly being re-evaluated and updated when necessary. In situations in which new GA (genetically altered) strains are developed, or modification to methods are used, work will initially be limited to small numbers of animals, with several sequential experiments undertaken to achieve publishable data. This will allow measurement of the inherent variation in outputs between experiments, which will help determine the number of animals required for subsequent experiments. Additionally, the continued development of animal alternatives, may lead to the reduction in animal numbers required in future experiments by being able to address specific scientific questions without the need for whole animals. Where appropriate we will use tools such as the EDA (experimental design assistant) to support our program of work.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will utilise animals up to optimum breeding age and not breed animals with unexpected background harmful affects. We will use multiple tissues from individual animals where it is possible to maximise outputs from animal procedures and minimise numbers of animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will typically use mouse models that have genetic modification of genes of interest. We will breed these mice to generate tissues and organs that we can then use ex vivo for downstream analysis after termination by a schedule 1 method (that is minor in terms of pain, suffering or distress).

To ensure mild outcomes of genetic modifications, whenever possible we will utilise mouse models using specific Cre-expressing lines that target conditional genetic changes to the testis. The reasoning behind this is that in humans male infertility causes no physical pain or discomfort. We thus expect that use of testis-specific Cre mice will similarly not cause pain, suffering or distress. We will also carefully choose Cre lines that are reported to have minimal expression outside of the testis. Our animal facility will visually monitor mice on a regular basis to ensure there are no unexpected effects caused by inappropriate Cre-expression in other tissues.

Where possible we will maintain mice as either knockouts or heterozygotes to reduce the numbers of mice that we need for breeding. In cases where we detect changes to animals that indicate any suffering above what we expect we will immediately consult the NACVO for advice and update our protocols for AWERB approval. In some cases we will breed animals for analysis by collaborating groups either in our local university (e.g. to analyse behaviour or neurophysiology) or within other locations.

Why can’t you use animals that are less sentient?

My research programme aims to understand gene expression control particularly within the testis. Reproductive systems are rapidly evolving, the further we move from mice as a model for humans, the less relevant our results will be for human fertility and infertility.

We have to use a mammalian model system. Some of the genes that we are investigating in mice/humans are not present at all in more distantly related model organisms (fish, insects, yeast and bacteria). This is the case for the RBMXL2 gene that is essential for mammalian spermatogenesis but not found in other vertebrates. Others genes like Tra2beta and SLM2 are present in other vertebrates, but have very specific patterns of expression in mammals that are not shared in more widely diverged vertebrates. This means that we have to use a mammalian model to investigate them in a context that is relevant to humans.

To reduce the amount of time we have to keep animal models in some cases we can use young mice. Such neonatal mice can be used for experiments that examine the early cellular stages of spermatogenesis, since despite not yet producing sperm they have some important cell types in spermatogenesis (such as cells undergoing meiosis, the special form of cell division that reduces chromosome numbers in sperm). However, in other

cases we have to use mice that are older than 6 weeks that produce sperm. We also need adult mice to monitor the effects of genes on testis structure (which is only properly developed in adults over 6 weeks). The Tra2beta and SLM2 genes are expressed in both testis and in the brain, so in some cases we want to monitor other aspects of whole animal biology typically monitored in adults (such as brain structure, electrophysiology on ex vivo brain tissue, and behaviour).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals, regardless of disease model will be checked regularly and supportive care will be provided to minimise distress or suffering and improve animal welfare. Most of our experiments will be carried out using ex vivo tissue, which will minimise the welfare costs for the animals.

We will monitor the health of our mouse strains using scorecards that record activity, appearance, body condition and weight, eyes, faeces and respoiration. Whenever we identify a welfare risk we will minimise its impact by working with the vet team and animal behaviour/welfare scientists we will carefully assess our animals' well being and level of comfort. We will cryopreserve inactive strains to minimise mouse usage, use colony management (basic backcrossing) to reduce genetic drift, and conduct regular genetic health analysis (SNP analysis) to ensure reproducibilty.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Alongside the guidelines listed below, I will also adhere to local AWERB standards for research animals, and where appropriate, support the development of new local standards for refinements discovered during the project licence.

- Code of Practice for Housing and Care of Animals Bred, Supplied or used for scientific purposes
- LASA Guidelines
- RSPCA Animals in Science guidelines
- UFAW Guidelines and Publications
- NC3R’s and Procedures with Care

I will consult with the Colony Manager to review genetic health, breeding practices and overall colony health and management at regular intervals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The local AWERB, NIO, NACWO, NTCO and Veterinary team regularly inform, and disseminate improvements and recent studies involving reduction, replacement and refinement, alongside external resources including (but not limited to); collaborators, peers, conferences and lab animal and animal welfare bodies.

During the 1, 3 and 5 year review of the project licence I will update on implementation or consideration of the 3Rs that has occurred during the previous period, alongside a review of the linked training plan, score sheets etc. in collaboration with the NACWO, NTCO, NIO and Veterinary team with a particular focus on refinements.

120. Transmission and Pathogenesis of Influenza and Coronavirus

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Influenza, Coronavirus, Respiratory, Transmission, Pathogenesis

Animal types	Life stages
Hamsters (Syrian) (Mesocricetus auratus)	adult
Ferrets	adult
Hamsters (Chinese) (Cricetulus griseus)	adult
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our research concerns viruses that cause respiratory tract infections of human and animals. This includes influenza viruses and coronaviruses like those that caused the COVID pandemic. We study three key interlinked areas: How does the virus pass from

one person to another (transmission), how much disease does it cause (pathogenesis) and can we find new ways to prevent or treat these infections (interventions).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Influenza is a respiratory disease of humans caused by a highly infectious virus that gives rise to annual outbreaks, and sometimes pandemics. Every year around 10% of the worlds' population are infected by a seasonal influenza virus and up to 650,000 people die. Influenza outbreaks are a major winter burden in hospitals and pandemics are costly and disruptive to society. Although there is an influenza vaccine, it has to be updated yearly to keep pace with evolution as the virus varies its antigens and evades pre-existing antibody responses. Ensuring a good match between the vaccine and the new circulating strain is a challenge that will be better met if we understood more about how the virus evolves and transmits between people. Mammalian models have been well established to provide valuable information about the prevention and control of influenza. The use of small mammalian models permits the study of host factors, viral determinants, and environmental constraints on the transmission of influenza viruses. Several small mammalian models, such as ferrets and mice are routinely employed in influenza studies.

The novel coronavirus SARS-CoV-2 was identified in December 2019 in China, with the virus spreading rapidly to other countries across the word. Since the COVID-19 pandemic started, over 6.5 million people around th world have died from the disease. Most people infected with the virus will experience mild to moderate respiratory illness and recover without requiring special treatment.

However, some will become seriously ill and require medical attention. Older people and those with underlying medical conditions like cardiovascular disease, diabetes, chronic respiratory disease, or cancer are more likely to develop serious illness. Anyone can get sick with COVID-19 and become seriously ill or die at any age. We have vaccines that have made a huge impact, but the virus is constantly evolving, throwing up new variants, and the vaccines will need to be updated and even modified to prevent virus spread. We have some drugs to treat the disease and infection but they are stil not optimal and there is much room for improvement. Animal models are essential to understand transmission, pathogenesis of SARS-CoV-2 and for development of therapeutic and preventative strategies.

What outputs do you think you will see at the end of this project?

We aim to understand:

1. How viruses that circulate in animals, and have not yet spread from person to person, might acquire genetic changes to become transmissible between humans, and what those changes are.

2. How influenza viruses and coronaviruses evolve in response to antibody pressure from a previous infection, and how vaccines might keep pace.

3. Why some influenza and coronaviruses cause more severe disease than others and how the host response to infection response influences disease outcome.

4. How influenza and coronaviruses might be controlled in the future by targeting viral or host functions that are required for virus replication or that influence the host response to infection response to virus infection, and whether the virus can evolve resistance to these therapies.

5. What route of transmission do influenza viruses and coronaviruses use, the relative importance of each route and whether we can develop new ways to block their spread.

6. As SARS-CoV-2 becomes a regular seasonal virus what will be the impact of co-infection of influenza and coronaviruses.

Outputs of this work will be disseminated to the scientific community through publication in peer reviewed journals and presentation at scientific conferences.

Who or what will benefit from these outputs, and how?

1. Defining genetic changes that allow an animal virus to become transmissible will inform surveillance and alert us to potentially dangerous viruses as they emerge in the field. Understanding the dynamics of influenza and coronavirus transmission will inform public health policy about how to deal with outbreaks, when people are most or least contagious, when to return to work. In addition, the basic knowledge gained about how influenza A viruses and coronavirus transmit through the air may be applicable to other respiratory infections caused by viruses or bacteria. Understanding how influenza viruses and coronaviruses evolve both within and between hosts during transmission will reveal the basis of seasonal drift and virus evolution and help us to predict better which variants are most likely to emerge each year. This would improve the accuracy of vaccine strain selection.

2. Understanding the basis of severe influenza and coronavirus disease and whether the host response to infection response is beneficial or harmful will inform development of new treatment strategies that interfere with the immune response. It will also help public health planners to model how they should prepare and respond to newly emerging influenza and coronaviruses.

3. Studying the dependence of the virus on host genes for its replication will lead to a new era of antiviral drugs from which the virus cannot escape. In addition, identifying

groups of people with different genetic make-ups who are particularly at risk or less susceptible to influenza viruses and coronaviruses will inform vaccination programmes. In the long term it may be possible to breed animals that are resistant to influenza and coronavirus infections to minimize future incursions of animal viruses into humans and outbreaks coming from a domestic animal source.

How will you look to maximise the outputs of this work?

Data from animal experiments will be shared with collaborators within the UK. The collaboration will yield benefits in both directions: experts in sequencing, evolution, and transmission dynamics modelling will gain access to detailed, well characterized in vivo samples, and the biological experiments will be analysed in a way that far surpasses what can be achieved by one group in isolation.

Outputs of this work will be disseminated to the scientific community through publication in peer reviewed journals and presentation at scientific conferences.

The PI is also an experienced science communicator, speaking to journalists in written media, radio and tv especially at crisis moments during outbreaks. They also sits on multiple advisory bodies from where they are able to relay the outputs to policymakers.

Species and numbers of animals expected to be used

- Mice: 1750
- Hamsters (Syrian) (Mesocricetus auratus): 2500 Ferrets: 850
- Hamsters (Chinese) (Cricetulus griseus): 700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Ferrets have been adopted as a ubiquitous and essential animal model for studying the transmissibility, pathogenesis, and pathogenicity of influenza viruses. Ferrets are the most suitable animal model for studying influenza virus transmission studies for the following reasons: 1) ferrets are highly susceptible to influenza viruses and can be infected with the majority of avian, swine, and human influenza viruses without prior adaptation; 2) ferrets display clinical signs and symptoms similar to humans, such as fever, sneezing, nasal discharge, and coughing, and these clinical signs may facilitate transmission; and 3) the physiology of the respiratory tracts and the distribution pattern of sialic acid receptors along the respiratory tracts are similar between humans and ferrets. These reasons strengthen the use of the ferret model as a gold standard to study human influenza transmission.

Mice have been used as a convenient animal model for influenza research due to their smaller size, fewer necessary resources, the availability of commercial reagents, and advanced techniques for gene manipulation. For these reasons, mice have been the most widely used animal model to study influenza pathogenesis and pathogenicity.

Since the emergence of the new SARS-CoV-2 virus early 2020, several animal models have been reported, and both ferrets and hamsters and some mouse strains are validated models. Several reports suggest hamsters are a superior model to the ferret for SARS-CoV-2 infection. The receptors that allow the virus to enter the lung, kidney and gastrointestinal tract are very similar in hamsters to those in humans. Previous studies with SARS-CoV-2 have shown there is efficient viral replication in the upper and lower respiratory cells, efficient transmission from inoculated animals (donors) to exposed animals (sentinels) via direct and indirect contact routes. Syrian Golden hamsters are the most common species used in the laboratory setting to study SARS-CoV-2, however Chinese hamsters have also been described as a good small animal model for studying SARS-CoV-2. Where possible and appropriate we seek to use the smaller animal model, which will enable slightly higher numbers and enhance the certainty of our data. As SARS-CoV-2 continues to spread at high rates in the human population, the virus is constantly evolving, with new variants periodically emerging. More groups of animals are necessary to compare transmissibility between two close variants, and to study immune escape of the new variants. Hamsters are also shown to support replication of influenza viruses and may be a good model in some cases for influenza.

Only adult animals will be used in our projects. Our research does not encompass aspects of the life course, and very young or very old animals would be expected to experience more severe disease.

Typically, what will be done to an animal used in your project?

1) Animals will be examined and screened by blood sampling, usually performed under light anaesthesia. All animals have a first nasal wash sample taken before infection. This is done while they are conscious and involves putting a small volume of liquid into their nose and collecting it as it runs out. .

2) Some animals will be given compounds and/or vaccines before or during their infection , this could be by injections or directly into their nose or in their feed.

3) Animals will be infected by putting virus into their noses or breathing in virus containing aerosols or by exposure to already infected animals.

4) More nasal washes are performed a maximum of twice daily for a duration of 21 days. The animals are conscious for these.

5) Animals are closely observed for clinical signs and weight loss.

6) To image the spread of virus in animals, chemical compounds that light up where the virus is may be given into their nose or by injection. The animal is under anaesthesia for this. This would happen maximum once per day for 7 days.

7) Animals may be placed in a chamber to measure virus they breathe out. They are conscious for this and spend no more than 10 minutes in the chamber., This may happen twice a day for up to 7 days.

8) Six weeks after infection, when animals are fully recovered, they may be re-infected via their nose or by exposure to other infected animals. They would then undergo the same series of sampling as above. All animals will be killed by a schedule 1 method or by exsanguination (including terminal bleed by cardiac puncture) under terminal general anaesthesia completed by a schedule 1 method at the end of the experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

We expect that the animals will show mild to moderate clinical signs such as lethargy, weight loss, transient fever, and sneezing, for no more than two weeks. Animals will be regularly monitored for signs of potential distress and will either be given appropriate treatment under veterinary direction if this is likely to be effective or, if showing signs of distress, will be killed without delay using a humane method. Analgesia and anaesthesia will be used where appropriate in order to minimise pain. Once experiments have ended animals will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Syrian hamsters – Mild (20%) and Moderate (80%) Level of Severity Chinese hamsters – Mild (20%) and Moderate (80%) Level of Severity Mice – Mild (20%) and Moderate (80%) Level of Severity

Ferrets - Mild (50%) and Moderate (50%) Level of Severity

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To study the processes of transmission and pathogenesis of respiratory viruses it is unavoidable that we use some animals because only in the whole animal are all the components of immune system and different cell types present. In vivo respiratory tract cells are organized in a functional pattern that affects the way the virus infects and propagates. Temperature gradients set up across breathing airways whereby noses are cooler than lungs, and the differential display in nose and lung of a different proportion of receptors for influenza viruses and for coronaviruses profoundly influences the initiation and progress of infection. It is not possible to mimic these factors in vitro at present.

Which non-animal alternatives did you consider for use in this project?

Using previous NC3Rs funding we have extensively explored the use of sophisticated cell culture systems, including primary differentiated respiratory airway cells of humans and of some animal species.

To better measure the airborne transmission of SARS-CoV-2 and influenza virus, we devised a machine which allows us to study viruses travelling through the air as they do during the transmission process. We find that using this exposure chamber and tube will ultimately enable us to reduce the number of sentinel animals we use. In order to validate and promote this novel in vitro system, we continue to work with animals to establish the parameters we should be able to measure in the new systems and to show that our results are in line with those of other labs that continue animal use.

As part of our research, we provide data from our animal work to research groups that model transmission of SARS-CoV-2 to reduce number of animals that would be used in the future, and we use modelling based on cell culture work to study the kinetics of the viruses.

Why were they not suitable?

The sophisticated cell culture systems do not currently include an immune component and yet we know that immune cells can contribute to the detrimental and damaging effects of the virus that is often a feature of severe virus infections.

The exposure machine does not allow us to study immune response and pathogenesis in sentinels infected by airborne route. Also, in sentinel animals, respiratory tract cells are organized in a functional pattern that affects the way the virus infects and propagates. Temperature gradients set up across breathing airways whereby noses are cooler than lungs, and the differential display in nose and lung of a different proportion of receptors for influenza viruses profoundly influences the initiation and progress of infection.

Modelling based on cell culture is limited because it does not take into consideration factors like the immune response and physiology. Models of transmission based on animal studies are being developed, but at the moment they are not accessible.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Group sizes for ferrets are as small as possible to still allow meaningful analysis of data. For example, using 4 animals per group, a number that is widely used in the peer reviewed literature from our own and other laboratories, it is possible to say with statistical certainty that there is a significant difference in transmission of two viruses, if scores of 0/4 and 4/4 are obtained. In our currently funded grant to study virus evolution, we are working alongside mathematical modellers to increase the amount of information from each experiment in a way that we hope will increase the robustness of the findings and enable us to continue to use these small group sizes with statistical confidence.

For mice and hamsters, group sizes of 4-10 animals are used. Our experience has shown that this number allows reproducible results between individual animals yielding robust values for statistical analysis. The group size is in line with published studies in the field.

Where possible our experimental design is such that experiments can be reported according to ARRIVE guidelines. This is applicable for testing the effects of compounds in mice. However, during transmission and pathogenesis experiments because group sizes are smaller and safety and experimental protocols require that we handle infectious animals in a specific order, randomization during handling is not possible.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experimental design is performed with the help of the NC3Rs EDA. Group sizes are calculated to ensure that animal numbers are kept to a minimum whilst allowing statistical power of the study to be maintained.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In vivo imaging will also be introduced where possible using genetically modified viruses that express enzymes that glow, marking out the location of the virus in the animal’s body. This will allow longitudinal study of the same animals and minimize the necessity to humanly kill animals at intermediate times for readout of lung viral loads.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will utilize three different mammalian species as models for the transmission and pathogenesis of influenza and SARS-CoV-2 virus in humans. These are ferrets, mice, and hamsters.

Ferrets are considered the best model for human influenza. Importantly ferrets express the same receptors in their airways as do humans so are susceptible to infection with clinical strains of human influenza viruses. Influenza in ferrets results in clinical signs of lethargy, sneezing, raised temperature and nasal discharge that reflect the outcome in humans. Avian influenza viruses, such as the highly pathogenic ‘bird flu’ viruses, do not transmit in ferrets reflecting what is observed in the field with humans. Other animal models do not accurately reflect human influenza. Mice express different receptors than humans, and are naturally at a higher temperature, which may favour conditions for replication for avian rather than human influenza viruses. Mice can be infected with some but not all human influenza viruses and most strains used in other labs have been prior adapted for growth in mice. Only very rarely has transmission of influenza viruses been reported between mice.

We use mice to study influenza and SARS-CoV-2 pathogenesis because they are a well-established small animal model to measure the balance between immune response and virus replication that determines outcome of infection. Mice that are genetically altered allow us to investigate the role of individual genes in outcome.

Hamsters (Syrian and Chinese) can be infected by influenza and coronaviruses. Previous studies have shown there is efficient viral replication in the upper and lower respiratory cells, efficient transmission of inoculated donors to sentinels and clinical symptoms such as weight loss, ruffled hair coat, hunched back posture and rapid breathing. For SARS-CoV-2 several studies including our own suggest that hamsters are a superior model to ferrets. Hamsters are easy to handle and reagents are more readily available that can support immunological studies for vaccine development. The smaller animal model enables slightly higher numbers and enhances the certainty of our data. Hamsters can also be used for influenza research, and this may be appropriate for some of our studies.

Why can’t you use animals that are less sentient?

Our primary purpose is to understand transmission of respiratory viruses and relate this to human infections. We need to use animals that become infected in the respiratory tract

and pass these viruses from one to the next through the air. Our transmission experiments require the animals to breathe normally in the air. Although fish can be infected by influenza virus, they clearly would not transmit virus in a way relevant to humans. we also require the infected animals to be active, anaesthetics supress this and modify the breathing patterns. We also expect the animals will have a mature immune system so that the balance between host immunity and pathogen reflects the human disease we are modelling as far as possible. Non-protected animals (e.g. invertebrates such as insects, decapods, nematodes) or less sentient animals (e.g. zebrafish) cannot be infected by human coronavirus and are rarely infectable by influenza viruses but that model is not relevant here.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal suffering is minimized by using the lowest dose of virus to achieve the aims of the experiment. Low volume inoculation into the nose rather than trachea avoids severe lung disease.

We have spent some time refining the way we handle animals during our experiments: animals are nasal washed conscious rather than under general anaesthesia as most labs do, so they are moving about straight away after the procedure therefore reducing any distress caused and avoiding any harmful build up of anaesthetic in their bodies. In addition, they are given rewards/treat post procedure. During the acclimatisation period they receive lots of handling to prepare them for the procedures that may take place.

The virus strains we use are not expected to induce severe disease. We do not use highly pathogenic avian influenza viruses (‘bird flu’) in our work. None of the viruses we use are associated with neurological disease.

None of our protocols are severe. Infected animals will experience mild to moderate respiratory illness and recover without requiring special treatment. We will monitor animals no less than three time each day to ensure they are not experimenting more severe disease. In the case an animal experiences weight loss of 20% for hamsters and mice or 15% for ferrets from its weight before the procedure or exhibits symptoms of distress and/or pain, it will be removed from the experiment and euthanized without delay. In the case of the animal expressing any unexpected signs of adverse effects (e.g. laboured breathing), gastrointestinal disease, vomiting and diarrhoea, it will be continuously monitored and in case the symptoms are not alleviated within the next 24 hours, the animal will be withdrawn from the experiment and euthanized.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The Laboratory Animal Science Association (LASA) guidelines and NC3Rs guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly visit the National Centre for the Replacement Refinement and Reduction of Animals in Research website for updates and appropriate courses. The establishment has an active 3Rs programme and always circulates updates from NC3Rs promptly. Also, we have registered for regular email updates of the NC3Rs newsletter.

121. Assessment of the Immune Response to an Autogenous Vaccine against Streptococcus Dysgalactiae in Sheep

Project duration

3 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Vaccine, Sheep, Lambs, Welfare, Streptococcus dysgalcatiae

Animal types	Life stages
Sheep	pregnant, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to measure the immune response of a veterinary autogenous vaccine being used on a sheep farm to prevent a common infectious joint disease of young lambs "Joint Ill", caused by a bacteria Streptococcus dysgalactiae.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Streptococcus dysgalactiae infectious arthritis (joint-ill) is a common condition of new born lambs that causes chronic pain and suffering to many lambs in the UK each year; as well as significant economic loss to the farming industry through lamb deaths and reduced growth rates. The disease will often occur despite farmers best efforts to prevent it, although administration of antibiotics is practiced in an effort to protect the lambs. Recent research has shown that the bacteria is commonly found in the ewe and the farm environment and is therefore difficult to prevent exposure of the neonatal lamb to the organism. A vaccine, which is given to the ewe in pregnancy could provide immediate antibody protection to the neonatal lamb through colostral transfer. Under UK legislation, veterinary practitioners can prescribe an emergency autogenous vaccine made from bacterial pathogens isolated from the flock when no licensed vaccines are available to control diagnosed disease. However, only clinical outcomes (change in the number of joint ill cases) can be measured by the veterinary surgeon. This project aims to work with such a veterinary surgeon to also examine the immunological response of the vaccine in the flock by measuring blood and colostral antibody levels in a subset of the sheep flock which is already planning to vaccinate.

What outputs do you think you will see at the end of this project?

We expect to be able to estimate the clinical and immunological effectiveness of veterinary vaccine against Streptococcus dysgalactiae in lambs and ewes. This will be new information; such data have never previously been reported. The information would be published in a scientific paper and would be shared with fellow veterinary professionals and the pharmaceutical industry through scientific publications and stakeholder meetings.

Who or what will benefit from these outputs, and how?

1. There would be immediate benefit at the conclusion of the work for the farmer and his flock that the study is being undertaken on. They will be informed immediately whether the vaccine programme was effective in stimulating a protective immune response in their flock and whether there is economic and animal welfare benefits to the flock of continuing the vaccine programme.

2. As the vaccine is produced by commercial company for each flock on an individual farm basis, the veterinary surgeon for this farm and other veterinary professionals could use this knowledge of vaccine efficacy to advise and implement similar vaccine programmes in other flocks under their care. This benefit could be realised within 5 years of the conclusion of the study.

3. The project aims to reduce the need to use antimicrobials in food producing animals through the use of vaccine to control "Joint Ill". This benefit could be seen within 5 years of the project being completed.

How will you look to maximise the outputs of this work?

Outputs will be maximised by

1) Preparation of a scientific publication

2) Communication of results through veterinary and farming meetings.

3) We will collaborate with the funders of the study to ensure dissemination of results of the study.

Species and numbers of animals expected to be used

Sheep: 500 ewes will receive the vaccine. A subset of these vaccinated ewes (200) and 200 unvaccinated ewes will be blood sampled. 400 lambs will be blood sampled.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will be using pregnant sheep pre and post vaccination because the disease only occurs in sheep and the vaccine is given in pregnancy in order that antibody protection is provided to the lambs in the colostrum.

We will also blood sample lambs between 24 hours and 7 days old to measure the uptake of antibodies from their mother's colostrum

Typically, what will be done to an animal used in your project?

The flock consists of approximately 1000 ewes. The ewes will be randomised to a vaccinated and unvaccinated group such that half of the flock will receive 2 doses of the vaccine 3 weeks apart. The unvaccinated group will be left untreated. A sub-set of 200 ewes from each of the vaccinated and unvaccinated groups will be blood sampled twice (pre and post vaccination). We will also collect a colostrum (milk sample) from each of these ewes as soon as she lambs. The collection of the blood samples is a very brief and minimally invasive procedure and normally takes less than one minute per ewe.

We will collect a single blood sample from 200 lambs born from each of the vaccinated and unvaccinated groups, between 24 hours and 7 days old. The collection of the blood samples is a very brief and minimally invasive procedure and normally takes less than one minute per sheep.

It is normal husbandry practice on sheep farms to remove a small volume of colostrum from a freshly lambed ewe to ensure she is producing colostrum for her lamb. Therefore, the collection of this colostrum for antibody testing is not a regulated procedure under ASPA and does not require a Home Office Licence.

What are the expected impacts and/or adverse effects for the animals during your project?

The vaccinated ewes will only experience very brief (less than one minute) discomfort during vaccine injection.

The blood sampled ewes and lambs will only experience very brief (less than one minute) discomfort during the collection of the blood sample

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity for both the procedures is classed as mild

What will happen to animals at the end of this project?

- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The animals being sampled are sheep and lambs from a sheep flock who will be receiving vaccination for the disease. Therefore, it is only these sheep that we are able to study. The disease does not occur in any other species.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives.

Why were they not suitable?

There are no non-animal alternatives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used unpublished pilot ELISA data from a similar vaccine study in sheep in Norway.

We have considered the size of the flock being vaccinated and the number of animals in the flock.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used a statistical sample size calculation tool Open Epi http://www.openepi.com/Menu/OE_Menu.htm

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot data and power calculations (see above)

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Sheep will be used in the study; the procedures are both brief and mild and should only cause transient discomfort to the animals.

Why can’t you use animals that are less sentient?

The disease under investigation only occurs in sheep, and the vaccine under investigation is only used in sheep.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The vaccine manufacturer's reccommended safety test of the vaccine will be conducted on a small number of sheep prior to study commencement.

All animals will be monitored post vaccination and sampling for any adverse effects, should these occur veterinary attention will be sought.

All procedures will be carried by a Home Office Personal Licence holders who are also veterinary surgeons.

We will ensure handling facilities at the farm are satisfactory and use sufficient trained staff to handle the sheep safely.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

ARRIVE guidelines will be used for study design and reporting.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The project licence holder will stay informed about advances in 3R's through engagement with the National Centre for Replacement Reduction and Refinement of Animals in Research Website and through seminars and information disseminated through the research institution where the project licence is held.

122. Effects of Metabolic Stress on Tissues and Tumours

Project duration

5 years 0 months

Project purpose

(a) Basic research

Key words

early-life, growth, metabolism, brain, cancer

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overarching aim is to identify the cellular and molecular mechanisms by which growing brains, brain tumours and other tissues such as the skin respond to metabolic challenges.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Understanding how growing brains, brain tumours and other tissues such as the skin respond to metabolic stresses such as malnutrition will provide important new knowledge. This will ultimately shed light on the molecular and cellular mechanisms underlying the clinically relevant processes of fetal growth restriction, brain sparing and brain cancer. In

the longer term, we anticipate that information about these mechanisms, gained from animal models, will provide valuable new insights that can be used to design new therapeutic approaches for the treatment and management of fetal growth restriction and brain cancer.

What outputs do you think you will see at the end of this project?

The expected outputs of this project are threefold:

1) Advances in fundamental knowledge via the identification of new cellular and molecular pathways that regulate the adaptation of normal and tumour tissues to metabolic stresses. This will facilitate the identification of the key specializations and vulnerabilities of different growing organs during health and disease. We aim to disseminate our findings to basic and clinical researchers in our own and other fields, as well as more widely in the public domain, by presentations at national and international meetings and publications on preprint servers and in peer-reviewed journals.

2) Production of metabolic disease and cancer models as well as other technical resources such as protocols and analytical tools for metabolomics. These valuable benefits will be made available to the academic, clinical research and pharmaceutical industry communities

3) Identification of candidate genetic targets and drugs that may, in the longer term, be developed by the pharmaceutical industry and clinicians to treat specific types of human cancer and metabolic disease.

Who or what will benefit from these outputs, and how?

Dissemination of our new biological findings and technical resources via conferences, preprint servers and peer-reviewed publications (see above) will benefit the global scientific research community both during and after the project. These findings will similarly benefit clinical researchers and the pharmaceutical industry, especially where they include new animal models of metabolic disease and cancer. These particular clinical and industry benefits are likely not to be fully realized until after completion of the project and are anticipated to have enduring long-term impact.

How will you look to maximise the outputs of this work?

We will maximise the outputs of our work by the timely publication of primary research papers and presentations at large international meetings and smaller more focused workshops. Not only will the positive results be communicated, but where it is useful for other researchers, unsuccessful approaches will also be highlighted.

We collaborate both internally and externally and this provides another avenue to share details of approaches that were suboptimal and information about how they may be improved.

Where it is appropriate, we will release unreviewed versions of our work on preprint servers such as bioRxiv and Wellcome Open Research to ensure rapid dissemination to the global scientific community.

Species and numbers of animals expected to be used

- Mice: 14,800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our aim is to identify the cellular and molecular mechanisms by which growing brains, brain tumours and other tissues (such as the skin) respond to metabolic challenges. These processes involve key metabolic interactions and signalling between multiple tissues that cannot currently be recapitulated in cell or organoid culture systems. It is therefore essential to use animal models and tissues derived from animals. In particular, the generation, breeding and analysis of genetically altered animals is necessary to test the functions of specific genes. It is necessary to work with mice as they have a physiology, anatomy and pathology that more closely resembles that of humans than other commonly used animal models, such as nematode (Caenorhabditis elegans), fruit fly (Drosophila melanogaster), or zebrafish (Danio rerio). Mice (Mus musculus) also offer the advantage of sophisticated genetic tools that are necessary for the design of the precise functional experiments required to unravel complex molecular mechanisms. Use of the embryonic, fetal, postnatal and adult life stages of mice are necessary to understand the causes and consequences of fetal growth restriction in response to metabolic challenges in tissues such has the brain. To understand how metabolic stresses impact upon the brain cancers glioma and glioblastoma, it is necessary to study postnatal and adult life stages as this is when tumour growth is manifested.

Typically, what will be done to an animal used in your project?

The majority of regulated procedures are of mild severity and involve the breeding of genetically altered animals, often with minor interventions such as ear clipping to allow genotyping. Typically several hundred matings of genetically altered mice will be performed each year. These will be used either to maintain specific genetic lines or to produce experimental animals of a specific genotype for basic research purposes using procedures of moderate severity. The animals generated for basic research purposes will mostly be used for dietary and metabolic models and for brain tumour models.

Typically, dietary and metabolic models will be used to study aspects of fetal growth restriction such as brain sparing, skin development, and their sequelae. In this case, protein or other components of the diet will be varied specifically during pregnancy. The

offspring animals at fetal, postnatal or adult stages will then be humanely killed and their tissues examined by immunohistochemistry and/or other analytical methods. Protein or other components of the diet may also be varied at other stages of development and adulthood in order to define the critical periods of nutrition for particular functions of the brain, skin or other organs.

Typically, for brain tumour models, animals at postnatal stages will receive intracerebral injections of glioblastoma cells, or substances designed to alter gene activity, in order to induce brain tumours. To develop novel anti-cancer therapeutic regimens, drugs are then administered with the aim of altering brain tumour growth and metabolism in these animals during postnatal and adult stages. Tumour growth is typically monitored by non-invasive imaging methods such as bioluminescence.

Subsequently, animals are humanely killed and their tissues analysed by multiple methods such as immunohistochemistry and metabolomics.

All animals will be closely monitored following regulated procedures, and anaesthetics, analgesics and/or other ameliorative procedures will be used as appropriate. In all cases, animals will be humanely killed if there are signs of pain, distress, suffering or weight loss above the agreed limits. The project will use the minimum numbers of control and experimental animals that are compatible with statistically valid conclusions.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected adverse effects on the wildtype and genetically altered mice in this project are a moderate loss of body weight and the formation of tumurs in the brain or in superficial tissues such as the skin. Typically, for dietary and metabolic models used to study fetal growth restriction there are no adverse effects but if weight loss were ever to exceed the limit on this licence (20% of body weight), then animals will be humanely killed. For brain tumour models, we do not expect metastases but for superficial tumours this may occur in some cases. Adverse effects on weight loss or abnormal behaviour can develop with brain tumour models but if these exceed the agreed limits or any significant signs of pain, distress, or suffering are observed, then animals will be humanely killed. In rare cases, dependent upon which gene is being altered, genetically altered mice may also develop adverse outcomes in the absence of any additional procedures. If any significant signs of pain, distress, or suffering are observed in these genetically altered animals, then they will be humanely killed. The maximum expected level of severity for any procedure conducted within this project is moderate and follows strict guidelines in accordance with the Home Office. At the end of procedures, all animals will be humanely killed by an approved method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Breeding and maintenance of genetically altered animals with sub-threshold severity is anticipated to account for ~65% of all animals used in this project because it can take several generations in order to generate the complex genotypes required for our research. Rarely, breeding of genetically altered animals will fall into the category of mild severity (<5% of these animals). The remaining ~35% of animals will undergo basic research procedures such as diet variation and tumour induction, which are both categorized as moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is necessary to use animals to study the growth of organs and tumours because this process is subject to regulation inside the body from numerous other tissues and circulating hormones. It is not yet possible to recreate this level of biological complexity or to mimick accurately the interactions between different organs using cells or organoids grown in a petri dish. Therefore, studies in the context of the whole intact animal are needed to identify metabolic and anti-cancer therapies that will ultimately be meaningful for human clinical studies.

Which non-animal alternatives did you consider for use in this project?

The insect Drosophila and also mammalian cells grown in a Petri dish.

Why were they not suitable?

We can gain a certain amount of valuable information from the use of non-protected animal alternatives such as the insect Drosophila and also from mammalian cells grown in various 2D or 3D culture models. Accordingly, both of these non-regulated models do form a significant part of the discovery phase of the proposed project, generating plausible and focused hypotheses that we can then test in mice. For example, we used Drosophila to identify an intercellular signalling pathway that may be conserved in mammalian brain sparing. We also used human glioblastoma cells cultured in a petri dish to identify some candidate drug combinations with potential anti-tumour effects.

Nevertheless, neither Drosophila nor mammalian cell culture models are substitutes for all animal experiments as they cannot recreate the complexity of interactions between numerous different cell types in the mammalian brain, and between the mammalian brain and other tissues in the body. For example, in the case of brain tumours, many potential anti-cancer drugs work well with gliobastoma

cells in culture but in vivo they are rapidly inactivated or they fail to cross the cellular barrier between the blood and the brain. We therefore anticipate that only a subset of all of the mechanisms identified in Drosophila or in cell culture will turn out to be important in vivo in mammals, thus making it essential to test the relevance of these mechanisms in mice.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals required are based on previous usage, and the numbers of breeding lines that we are currently maintaining. We carefully design experiments to be sure that we use the minimum number of animals required to give clear scientific answers. Typically for dietary and metabolic models used to study fetal growth restriction, 5-6 animals per group are required to achieve statistically significant results. For brain tumour models, where tumours are variable between animals, typically 10- 20 animals per group are required to achieve statistically significant results. The sex of individual animals is recorded in all of our studies and both sexes are used.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We combined regular consultations with statisticians and our own past experience of actual effect sizes in order to calculate the sample sizes used in the animal work on this project (see above). Design will be based on PREPARE guidelines and sample sizes may be set using power analysis, generally using a significance level of 5%, a power of 80%, and a least practicable difference between groups of 20%. Otherwise, we will use the minimum number of animals to provide an adequate description on the basis of previous experience (our own and from the literature). We will also take advantage of online tools, including the NC3Rs Experimental Design Assistant, randomization tools for allocation of animals to groups, and the ARRIVE 2.0 reporting guidelines to help us to design optimal experiments with the minimum animal numbers required to achieve statistically significant results. In many cases involving analysis at fetal and postnatal stages, an efficient

statistical design uses mixed-effect models that account for different variances within a litter versus between litters. In addition, when testing new drugs or new dietary or tumour models, we will first perform small-scale pilot studies before embarking on properly powered experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Wherever possible, we will always use non-protected animals or other alternatives. More specifically, the Drosophila models (e.g brain sparing during nutrient restriction) and mammalian cell culture systems (e.g human glioblastoma cells) that are routinely used in our laboratory provide an important driver and hypothesis generator for this project and they are integral to its success. These models allow us to replace some of the mouse experiments that would have been conducted during the initial exploratory phase of hypothesis building. Another important aspect of minimising the numbers of animals is the efficient breeding and maintenance of genetically altered mice, which will constitute the majority of regulated procedures in this project. In this regard, we regularly review our mutant and transgenic lines and maintain only a minimum number of mice. We also freeze sperm and/or embryos to archive mouse lines. Thus, we will only maintain breeding lines that we are actually using in on- going experiments. In addition, the number of breeding animals is kept as low as possible by sharing mice lines between a number of other labs.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use mouse models of fetal growth restriction in response to dietary interventions. It will also use mouse cancer models of superficial and brain tumours. These models are well established and have been extensively characterised in many labs around the world.

In the case of mouse dietary and metabolic models, we are using dietary interventions as these provide a good model for malnutrition, the most common cause of fetal growth restriction worldwide. In addition, dietary interventions are less harmful than alternative models of fetal growth restriction that involve exposing dams to low oxygen tension (hypoxia) or performing surgical manipulations such as bilateral ligation of the uterine arteries.

In the case of mouse cancer models, we have chosen to use well established methods that involve superficial or intracranial injection of cells or other agents (e.g virus or plasmid) to induce brain tumours. These procedures have been extensively characterised and refined over the years to minimize pain, suffering and distress. When assessing tumour burden, the principles set out in the NCRI Guidelines for the Welfare and Use of Animals in Cancer Research will be followed. Tumour bearing animals will be humanely killed at a specific timepoint that will be earlier or equal to the time when they show adverse symptoms. For superficial tumour models, harm will be minimised by frequent monitoring to ensure that animals are humanely killed if they exhibit any adverse signs of pain, suffering and distress, and before the tumours reach the size limit stated in this licence. For mouse brain tumour models, tumour size is difficult to measure accurately in a non-invasive manner. These animals will therefore be frequently monitored for weight loss, body condition score, and for signs of pain and distress including altered respiration rate, locomotor defects, huddling or hunched posture, and piloerection of the coat.

Why can’t you use animals that are less sentient?

We will use the fruit fly model Drosophila (as well as mammalian cell culture) to replace some of the mouse procedures that would otherwise be required to deliver the objectives of this project (see Replacement section). However, mice rather than less sentient animals need to be used for this project as, unlike Drosophila, they share very similar genes, metabolism and physiology with humans and so are an appropriate model for providing insights relevant for human diseases and, ultimately, for developing human therapies. Mice have well-established laboratory procedures and advanced genetics, which both help to expedite research progress. In all cases, animal suffering will be minimised by following strict guidelines in accordance with the Home Office and by regularly monitoring animals in consultation with a named animal care and welfare officer and a named veterinary surgeon.

For dietary and metabolic models used to study fetal growth restriction, the majority of animals will be humanely killed during the fetal life stage, with a minority being humanely killed at postnatal or adult stages. For brain tumour models, the majority of animals will undergo cerebral injections to induce tumours at the postnatal rather than the adult life stage. Terminally anaesthetized animals will be used for several procedures such as intra-vital imaging, electrophysiology, exsanguination and perfusion fixation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will minimise the adverse effects associated with genetic alterations by using, wherever possible, inducible or conditional alleles to delete gene activity from specific tissues rather than from the entire mouse. To minimise stress during breeding and maintenance, we will follow best practice guidelines and implement local refinements of husbandry such as environmental enrichment and sufficient amounts of nesting material.

On generation of a new line, we will minimize suffering by ensuring increased observation and monitoring until a detailed phenotypic analysis is accomplished. If any welfare implications are identified, they will be acted upon and refinements considered in consultation with the NVS and NACWO.

I will ensure that all personal licence holders working on this project are made aware of and receive training in the published best practice guidance for animal monitoring, aseptic surgery, post-operative care and the minimization of pain (see section below). Most of the genetic, dietary, and other manipulations as well as the administration of gene activity inducers/repressors or other agents are standard and therefore previous refinements from the literature will be used. If, however, there is insufficient information available, new procedures or new drugs/substances will be screened in small- scale pilot studies to obtain indications of the minimum dose and exposure time that is likely to be effective, thereby minimising suffering.

In all surgery, analgesia will be provided according to published best practice and also advice from the NVS and NACWO. Good aseptic surgical techniques, heat & fluid therapy will be provided. During the project, I will consult with our NVS to optimise and improve surgical methods and communicate this effectively to the personal licence holders working under this project licence.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Experiments will be planned in accordance with the PREPARE guidelines [1]. For surgical procedures, we will follow the guidelines set out in LASA Guiding Principles on Preparing for and Undertaking Aseptic Surgery (https://www.lasa.co.uk). With regards to the experiments in brain tumour models, we will adhere to the Guidelines for the Welfare and Use of Animals in Cancer Research [2]. In addition, I will also keep myself and the personal licence holders working under my project licence updated with the latest refinement advancements in the use of dietary, metabolic and tumour models in mice by attending conferences, reading journal articles, collaborating with experts and monitoring the NC3Rs website and Resource Hub (https://nc3rs.org.uk/3rs-resources).

1. Smith, A. J. et al. PREPARE: guidelines for planning animal research and testing. Lab Animals 52, 134–141 (2018).

2. Workman, P. et al. Guidelines for the welfare and use of animals in cancer research. Br J Cancer 102, 1555–1577 (2010).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep up to date with the latest 3Rs developments via the NC3Rs website (https://www.nc3rs.org.uk), complemented by information we obtain from regular

newsletters prepared by our animal facility and mandatory annual meetings for all project and personal licence holders. In addition, there will be regular meetings between the project licence holder and all personal licence holders working on this project to ensure efficient communication and discussion on the 3Rs and any amendments or legal changes to the project licence and its implementation.

123. Elasmopower: Investigating the Effects of Emf on Elasmobranchs

Project duration

5 years 0 months

Project purpose

- Basic research
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Electromagnetic fields, Behaviour, Physiology, Elasmobranch, Windfarm

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to determine whether Electromagnetic fields (EMFs) generated by the subsea power cables of Marine Renewable Energy Devices impact shark and ray (elasmobranch) behaviour and physiology. This research will provide solid information to integrate into future planning applications and consents assessing how these cables may impact on these species (Ecological Impact Assessments (EIA)). The outcomes of this research will contribute to advancing mitigation methods, and feed into the offshore planning and consent process.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Elasmobranchs are apex predators around the seabed, they play a key role in the food chain of these ecosystems. It is therefore crucial to understand how they interact with subsea cables and what potential impacts encountering such devices may have. As a benthic species spending the majority of the time on the sea floor, they are highly likely to encounter subsea power cables in their natural habitat ranges and be exposed to increased man-made EMFs for an extended period of time. It is important to observe and understand how these EMFs could impact their behaviour and physiology.

The information obtained from this study will contribute to the current lack of knowledge on the impacts of EMF on marine species and will aid in future planning and consent processes and species management.

What outputs do you think you will see at the end of this project?

The primary output will be new knowledge about the impacts of EMF, at strengths measured around offshore wind farm export cables, on elasmobranch behaviour and physiology.

This information will be published in academic journals, an industry report, and widely disseminated to ensure inclusion in future studies and EIAs.

This project also hopes to refine husbandry techniques of these species, which remain very scarce, specifically for their use in research projects.

Who or what will benefit from these outputs, and how?

Government bodies, NGOs, and the renewable energy and fishing industries will benefit from this novel knowledge base, with the overall aim of improving the consenting process and associated EIAs.

Given the infancy of offshore renewable energy, and particularly research related to this field, any contributions made underpinned by robust scientific research is invaluable.

The information gained from this study will also contribute to the advancement of mitigation measures and conservation efforts for these commercially and ecologically important species.

How will you look to maximise the outputs of this work?

Close collaboration with researchers in the UK and the Netherlands, investigation and production of new information (peer-reviewed publications, conferences presentations) on the interactions of elasmobranchs and subsea power cables will help maximise the outputs of this project.

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

There is a current lack of knowledge on the potential effects of Electromagnetic fields on elasmobranchs, which are keystone species in the North Sea. As apex predators, they play a crucial role in the regulation of ecosystem interactions. Any impact on their behaviour and physiology will likely affect the dynamics of those ecosystems.

In order to fully understand the potential threat of electromagnetic fields, it is important to understand how they could influence key life stages. To ensure potential impacts are not overlooked, and to account for different sensitivities between life stages, both adults and later juveniles must be assessed during exposure to EMF.

Typically, what will be done to an animal used in your project?

Each animal will be placed in a 15m long tank, part of which will be exposed to low levels of EMF. The behaviour of the animal will be observed and recorded, which will allow for detection of potential changes in behaviour.

Additionally, small samples of blood will be taken from adults in order to analyse known physiological stress parameters.

What are the expected impacts and/or adverse effects for the animals during your project?

It is expected that EMF exposure will have no adverse effect on the animals. Mild effects on behaviour are expected such as changes in swimming behaviour, increased avoidance of the exposed area, and altered activity levels.

Short-term changes in physiological stress parameters may be detected during exposure, however it is anticipated that these will be similar to those detected through handling stress and quickly return to normal. Preliminary samples will be run in order to determine best practice for blood sampling, volumes, and assess test kits limits. These samples, as well as the comparison with the results from control individuals, will allow for changes due to handling be identified.

None of these effects are expected to cause long-lasting harm to the individual. Furthermore, these effects are anticipated to be acute in nature and organisms are expected to revert to their natural states within reasonably short time periods.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild severity is expected for all animals.

What will happen to animals at the end of this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This research aims to establish information on the impacts of electromagnetic fields on elasmobranchs. Currently there is a lack of information on basic elasmobranch behaviour, particularly around subsea power cables. This information is essential to model and predict the impacts of EMFs on these species. Therefore, the use of animals is essential to achieve the objectives of this project.

Which non-animal alternatives did you consider for use in this project?

N/A

Why were they not suitable?

The complexity of in vivo systems and the behavioural and physiological aspects of this study cannot be accurately reproduced in vitro, hence justifying the use of whole living animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals that will enable adequate results from both behavioural and physiological work has been chosen for this study based on previously published work.

Previously published behavioural studies on small spotted catsharks have used high numbers of replicates in order to account for high variability between individuals (88 and 66 individuals respectively in these two studies).

Throughout the project, power analyses will be conducted, and number of replicates reviewed periodically, and changes made accordingly.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The experimental design focused on ensuring that all data collected was robust and reliable thus reducing the need to run additional replicates. The experimental design has been categorised into behavioural and physiological trials whereby individuals used for physiological work will not be reused, nor have experienced EMF in prior behavioural trials. A minimum of 10 replicates for physiological analysis allows for the minimum number of samples to be attained accounting for assay error. The currently unknown duration of effects that may be attributed to EMF exposure, play a crucial role in the experimental design. To ensure accurate results are obtained, with minimal interference, the reuse of animals will be limited to behavioural trials, will not see a repeat of a given treatment, and only after a set period of time to avoid habituation. Behavioural data will be analysed each day to determine if fewer animals can be used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Behavioural information will be obtained from published literature to aid in the creation of baseline data which, if suitable, will reduce the number of baseline behavioural studies required as behaviours will be categorised and used to assess changes brought on by EMF exposure.

Preliminary studies will be conducted on 3 individuals of each species to assess if there are any lasting effects (>48h), behaviourally and physiologically, to EMF exposure. If the results from these preliminary studies show a lack of effect beyond a 48h period, then animals used for behavioural studies might be reused in additional trials to limit the number of new individuals needed. Those individuals used for physiological analysis via blood sampling will not be re-used, however periodical assessment will be undertaken whereby values obtained through blood work assays will be assessed for variation and robustness. If results show low variation and fall within the testable limits of the assays, then a power analysis will be conducted, and the number of replicates reduced to reflect the outcome of this test.

Numbers will be periodically reviewed and potentially reduced where possible and published literature will be reviewed as the project advances to stay informed of current best practices.

Statistical analysis will be conducted based on similar studies conducted in this field at our centre. Statistical analyses and experimental design have been discussed with statisticians.

For all aspects of this project, as part of good laboratory practice, we will produce an experimental protocol which includes:

- project and experimental objectives

- experimental description covering experimental treatments (both AC and DC treatments will be used as both types of cables are used around MREDs), sample sizes, identification of individuals to be used, duration, and type of experiment (behavioural or physiological).

- outline of planned analysis for data obtained, description of tests of significance to be made, outline of results obtained and assessment of variation of data and results from power analyses undertaken for each set of experiments.

In addition, excess blood samples collected during the project will be stored for later collaborative analysis. The main facility has limited analysis equipment. However, partner organisations and researchers have shown interest in expanding upon current haematological analyses.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will involve three non-protected, and non-endangered species of elasmobranchs as model species to reduce impacts on natural local populations: Small-spotted catshark (Scyliorhinus canicula), Thornback ray (Raja clavata) and a third ray species to be determined.

These species have established, yet limited, behaviour and physiological thresholds which will be compared against during the study, resulting in shorter experiments with lower overall animal numbers.

The EMF strengths utilised in this project replicate those measured at relevant cable sites in the North Sea. Both AC and DC cables are used around those windfarms and serve different purposes. At both sites, elasmobranchs have been found suggesting impacts will be minimal and can be concluded to be far below lethal levels.

All animals will be allowed a period of acclimatisation to experimental set-ups prior to commencement of experiments, in order to recover from the short period of handling and transfer to experimental tank. Experimental tanks will be subjected to the same conditions (temperature, salinity, dissolved Oxygen, pH) as holding tanks to avoid inducing unnecessary stress.

All protocols utilised throughout this project are categorised as mild. In behaviour experimentation individuals will have the option to remain out of the EMF which is localised to one portion of the experimental tank. Physiological studies, conducted via repeated blood samples over a 24h period, will be conducted in a continuous low-level EMF based on conditions at relevant cable sites in the North Sea.

All precautions will be taken to minimise suffering when applying procedures (careful handling, procedures conducted by technically qualified people, consultation of elasmobranchs experts).

Why can’t you use animals that are less sentient?

The three elasmobranch species are commercially and ecologically important in the North Sea and understanding how they interact with subsea power cables is key to their conservation. This project aims to use values measured live on the site of functioning subsea cables with 3 species adopting different behaviours and ecological niches, used to provide a comprehensive answer as to the impacts of EMF on elasmobranchs.

To date work has been conducted on the impacts of anthropogenic EMF on several marine groups including crustaceans, marine mammals, bivalves, and gadoids however there is minimal information on the impacts on elasmobranchs. Elasmobranchs are known to be electrosensitive, but the impact of anthropogenic EMF remains understudied and observing those effects in laboratory experiments is an important step in understanding future implications. To fully understand sensory thresholds, and physiological and behavioural changes in elasmobranchs when exposed to EMF the use of alternative species or terminally anaesthetised individuals will not be suitable.

Adults will be used at this stage but as the use of both adults and juveniles is imperative in understanding the potential differences in impacts at different life stages an amendment may be sought at a late stage to include juveniles.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be continuously monitored before, during and after the trials in order to minimise welfare costs. Contingency plans are in place if euthanasia is necessary under NVS guidance.

All procedures used have mild severity and previous scientific work has established robust and refined protocols for sampling blood in these animals.

The experimental arena (15m tank) is designed so that only a specific area (2x2m) is exposed to the artificial EMF. This allows the animal to remain outside of the impacted zone. All animals will be allowed to recover fully after experimentation, and once reviewed by NVS, will be released back into the wild.

All animals will undergo health assessments upon arrival to the facility and will be monitored for a period of several weeks before experimental use. Measures are in place and environmental options available for the animals to display natural behaviour while in holding tanks (shelter provided by different sized tubes, rocks and kelp for shelter and egg laying in the case of catsharks). This, combined with daily monitoring of behaviour in holding tanks allows for thorough checks and ensuring high standards of welfare. Animals will undergo minimal handling and blood samples will be taken. All animals will undergo a final health assessment before being released to the wild at the end of the project. All results obtained, both negative and positive, will be published.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Two volumes of elasmobranchs husbandry documents are available, which include peer-reviewed articles on different husbandry aspects, use of elasmobranchs in research, sampling methods, common pathologies observed in captive elasmobranchs and other relevant topics for this project.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will maintain focus on novel work being conducted and published in this field, regularly consult the relevant regulations and sources (i.e. NC3Rs resources) to keep on top of new advances in the 3Rs and review our protocols on a regular basis to refine the methodologies.

124. Development of New Treatments and Diagnostics for Kidney Injury

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

kidney injury, fibrosis, therapy, kidney biomarkers, inflammation

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to understand the key cell types involved in kidney injury and repair, how they interact and their distinguishing properties. By understanding these mechanisms and interactions we can manipulate them to prevent fibrosis and augment repair post injury.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chronic kidney disease (CKD) has been recognised as a leading public health problem worldwide. The global estimated prevalence of CKD is 13.4%, and patients with end-stage kidney disease (ESKD) who need renal replacement therapy is estimated to be between 4.902 and 7.083 million.

CKD is the loss of the ability of the kidney to filter blood over time; where the normal kidney tissue is replaced by scar tissue and some patients progress to end-stage kidney failure (ESKF) where dialysis and transplantation are the only treatments. CKD can be caused by numerous conditions with hypertension, diabetes, glomerulonephritis, obstruction of ureters and progression from acute kidney injury being the leading causes. CKD is an independent cardiovascular risk factor, present even with mild renal dysfunction, and in patients with CKD, cardiovascular disease (CVD) remains the leading cause of death. The progressive nature of CKD, resulting fibrosis and eventual ESKF places a significant burden on the NHS with total CKD costs of £1.45billion/year. Current management of CKD is based on controlling known risk factors e.g. hypertension and diabetes, which can slow progression but no treatment which targets fibrosis or specific disease specific pathological mechanisms are in clinical use.

Currently there are few things that can be non-invasively measured in blood and urine (biomarkers) to determine if you have CKD. A kidney biopsy, where a small piece of tissue is removed from the kidney, remains the gold standard but is purely for diagnosis of the specific condition causing CKD and is not without risk. Serum creatinine and protein in the urine are typically used to diagnose CKD initially by detecting a loss of kidney function and to monitor patients glomerular fitration rate (GFR). However both these biomarkers can be normal despite significant kidney damage and are sensitive to factors such as sex, frailty and ethnicity. Biomarkers of early injury and kidney health are urgently needed to prioritise patients and optimise treatment.

Scarring of the kidney affects the filters within the kidney that filter the blood and the cells that excrete and reabsorb essential nutrients. Scarring of the kidney is the final common pathway for all kidney diseases, regardless of what condition/disease caused the initial damage, and this scarring leads to kidney failure. The features you can see with a microscope in the tissue are excessive deposition of scar tissue which replaces normal tissue, the influx and activation of immune cells, tubular cell loss and loss of the small blood vessels.

Our recent research using state-of-the-art techniques such as single cell RNA sequencing and paired blood exchange has identified that monocytes (a type of immune cell) are recruited to the injured kidney and rapidly differentiate into a pro-inflammatory, pro-fibrotic phenotype. Furthermore, we have identified a novel macrophage phenotype that is

specifically present in the kidney during repair. We hypothesise that these novel monocyte and macrophage phenotypes may be exploited to ameliorate renal injury or promote repair. In addition we have used our expertise in small RNA sequencing to generate a miRNA atlas of miRNA expression during kidney during injury and repair which is free to use and has allowed us to use bioinformatic pipelines to identify key potential miRNA involved in kidney injury and repair and biomarkers which could be utilised as novel diagnostics.

The use of suitable models of renal disease has produced very successful translational results. For example, the use of angiotensin converting enzyme (ACE) inhibitors in CKD patients was informed from studies using the subtotal nephrectomy model as studies in preclinical animals demonstrated that the use of ACE inhibitors can inhibit progression of CKD. Utilising models such as the subtotal nephrectomy model also allows for study of how kidney injury impacts on the heart and vessels and increases the risk of CVD.

Finding effective targets for future therapeutics which can be translated from preclinical to clinical use depends on there being a deeper understanding of the molecular signals that modulate kidney injury and fibrosis.

What outputs do you think you will see at the end of this project?

The major outputs we expect to have at the end of 5 years will be:

1) Identification of a therapeutic intervention such as a novel drug, gene, miRNA, cell therapy or immunotherapy based intervention which has significant impact in appropriate pre-clinical models as measured by improved kidney function and reduced fibrosis. We would expect to publish these findings in 3-5 peer reviewed manuscripts.

2) New datasets. When we generate new datasets we will make them freely available to the research community to facilitate rapid dissemination and uptake of our findings. We have previously done this for our single-cell, bulk RNA sequencing.

3) Identification of potential new biomarkers of renal injury and kidney health which can identify the risk of progression and/or disease severity in pre-clinical models that we can then translate to patients using our biobank of human samples from patients with CKD.

Who or what will benefit from these outputs, and how?

The publication of papers and presentation of data to the community will have an impact on the scientific community in the short-term (5 years) of this project. This benefit will be achieved by continuing to publish in this field and moving what is known forward with new information. We will also attend conferences to present our findings over the course of the years to inform the community and also allow meetings and discussions with our collaborators.

Longer-term positive output from this project could have a impact on CKD patients. Patients with kidney disease and their families have a reduced quality of life and options such as dialysis are inconvenient and unpleasant. Patients with progressive kidney disease have very few therapeutic options prior to requiring renal replacement therapy in the form of dialysis and transplantation, neither of which are the perfect solution. Therefore the identification of novel drug, gene, miRNA, cell therapy or immunotherapy based interventions to slow or prevent disease progression or promote kidney repair would be a major advance in treatment. During this project we will seek to identify new targets which if were successful in pre-clinical models we could translate and we believe that these new treatment(s) could significantly reduce the economic burden on the NHS within 20 years. Furthermore, new treatments for CKD should allow patients to be healthier for longer enabling them to work longer and be less of a caring responsibility to others.

During this project is we are successful in identifying sensitive biomarkers for the risk of progression of disease and the severity this would be advantageous in the medium to long term (5-10 years) since we can optimise treatment earlier for these patients and as these patients are at high risk of CVD as their kidney disease progresses treatment at earlier stages would possibly prevent premature cardiovascular deaths.

How will you look to maximise the outputs of this work?

In order to maximise the outputs of this work we will seek to publish the results of all studies conducted under this project and where possible open access journals to ensure the widest dissemination of our research. For all publications we will use our social media accounts to share the data with the field and patient groups/charities.

For negative results we will as a minimum publish these as preprints and share the findings via our social media accounts.

Where possible we will seek to ensure we release accessible statements about our results specifically for the kidney patient community and their families.

Species and numbers of animals expected to be used

- Mice: 5000
- Rats: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We propose to use rats, mice and genetically altered (GA) rats and mice in order to achieve the objectives of this proposal. Rats and mice have been proposed to be used in this study as the majority of published data is available from these species and using mice and rats allows for the establishment of GA models.

In order to study chronic kidney disease an animal model has to be used as the initiation, progression and reversal involves several cell types and an inflammatory response which we are unable to replicate in cell culture. Aspects of the mechanism can be study in cells (in vitro) and in computer models (in silico) and this will be conducted when an option.

For certain studies rats are required to be used rather than mice for example to detail renal function/haemodynamics/imaging which can be limited in the mouse due to their small size and thus only in rat can we gain meaningful measurements. Mice will be used as there are appropriate genetically modified mice strains that can be utilised to address important mechanistic questions that are required to fulfil the objectives of this PPL. It can also be important to demonstrate that results found in one species are transferable to another and not specific to the species used. This is particularly important when looking for translational strategies. Furthermore, data obtained from these models in mice and rats have been demonstrated to be relevant to the human condition of CKD.

Typically, what will be done to an animal used in your project?

A typical animal used under this project will have

1) Induction of kidney injury (either surgical or by administration of a toxin)

2) Investigation of the injury mechanism (studied at protein, RNA and non-coding RNA level)

3) Either modification of genes OR introduction of a drug, and the effect on kidney injury assessed, typically this will improve fibrosis and, if appropriate, kidney function.

4) Blood and urine samples collected

5) Animal culled and processed to collect tissue, cells, blood which will be analysed and stored.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected adverse effects of animals during the project are:

Animals may experience wound pain from surgery and malaise lasting <72h and weight loss. This will be minimised by the administration of analgesics and group housing.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Under this project the expected severities would be:

For animals used under breeding and maintenance all animals would be expected to be mild severity.

For animals used under the other protocols the expected severity is moderate and all animals would be expected to be moderate severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Due to the complexity of kidney disease and the involvement of multiple cell types it is not possible to fully mimic kidney disease and repair fully by non-animal alternatives.

Which non-animal alternatives did you consider for use in this project?

Where possible before using animals we utilise several cell culture models that we have generated which can mimic parts of the process utilising for example tubular cells, inflammatory cells and fibroblast cell lines. With these in vitro studies we can carry out some of our mechanistic studies and evaluate the identified therapeutic cells/genes/non-coding RNA prior to carrying out studies in animals.

We now have the ability to data mine expression datasets from previous in vivo experiments carried out in our CKD models and from other researchers which are deposited in free to access public gene expression databases. Therefore we have extensive bioinformatic pipelines to examine any potential targets by in silico means rather than having to perform animal experiments.

We have a biobank of human CKD samples, we use these to screen for potential new biomarkers instead of using animals once they have been identified.

Why were they not suitable?

The in vitro models we have give us vital information about the mechanisms we are studying but they do not have the interactions with other cells which we know are vital for

the initiation, propagation and resolution of injury. Therefore these only allow us to examine a single snapshot of a complex mechanism which may not be relevant when considered at the tissue level.

Bioinformatic data mining allows for the identification of candidate genes/non-coding RNA however provides no insight into function. The function can be assessed in in vitro to see if these targets are high quality but their role can not be fully elucidated until the whole tissue and all cell types are present.

A human sample biobank is an excellent resource to use once we have identified new biomarkers which will come from the in animal modelling. This allows biomarkers to be tested in a large number of samples without the use of animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers have been calculated from previous PPL and from the planned experiments we have over the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For any study we identify the lowest appropriate group sizes for each group to give us appropriate statistical power. We use the NC3Rs experimental design assistant to help us improve the design of our experimental studies. We have robust randomisation and blinding protocols in place to ensure we are conducting high quality studies and we publish this detail in our papers. Animal genetic backgrounds are also considered when designing experiments to ensure we are controlling variability.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For our experiments we only generate the number of animals we need through efficient breeding practices and we freeze down sperm when the line is no longer being used and have it available to others.

We actively use historical tissue we carefully bank when we cull animals on procedure. This ensures we have good quality tissue and histological blocks available which allows

pilot studies for new avenues of research to be conducted without the use of new animals thus reducing the number of animals we use.

For untreated controls we store carefully archived tissue which we use rather than new animals were possible.

The inclusion of ultrasound and MRI imaging should in future allow us to refine our ability to assess fibrotic damage in the kidney and heart, leading to a reduction of the number of animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We propose to use rats, genetically altered rats and mice, and normal mice in order to achieve the objectives of this proposal. Rats and mice have been proposed to be used in this study as the majority of published data is available from these species and using mice and rats allows for the establishment of GM models.

For certain studies rats are required to be used rather than mice for example to detail renal function/haemodynamics/imaging which can be limited in the mouse due to their small size and thus only in rat can we gain meaningful measurements.

Mice will be used as there are appropriate genetically modified mice strains that can be utilised to address important mechanistic questions that are required to fulfil the objectives of this PPL. It can also important to demonstrate that results found in once species are transferable to another and not specific to the species used. This is particularly important when looking for translational strategies.

Furthermore, data obtained from these models in mice and rats have been demonstrated to be relevant to the human condition of CKD. In order to study chronic kidney disease an animal model has to be used as both processes involve several cell types and an inflammatory response which we are unable to replicate in cell culture.

For the surgical models of kidney injury we use two that cause asymptomatic kidney injury as the other kidney functions as normal. This prevents the animal from suffering any discomfort due to the kidney injury but provides important information about mechanisms. These models are used more often as these cause the least pain and suffering.

The functional models of kidney injury will induce kidney injury which may be progressive in nature. These are important models and have significant advantages over the asymptomatic models as they more closely resemble human CKD and data from these models is more clinically applicable. These models are essential for examining if newly identified cells/genes/non-coding RNA manipulation can improve kidney function and to understand the mechanisms by which kidney injury affects the organ and transmits to other organs. For these animals the experiments are carefully planned to use the lowest number of animals to have a statistically valid group number. Sham/Naïve mice will only be used when essential and where possible historical tissue will be used. The length of time the animals are on these protocols is carefully selected so that animal suffering/distress is as low as possible.

The inclusion of ultrasound and MRI imaging should in future allow us to refine our ability to assess fibrotic damage in the kidney and heart, leading to a reduction of the number of animals used. We will also use non-invasive ultrasound insonation to augment release of miRNAs into the blood and urine which will increase the sensitivity of this type of biomarker.

For substance administration we will use the most appropriate route which causes the least animal distress/suffering. For example using where possible osmotic minipumps to avoid repeated administration of substances.

Why can’t you use animals that are less sentient?

CKD is a disease of middle age and later life so using immature life stages would poorly model the mechanisms we seek to understand. Data obtained from mice and rats have been demonstrated to be relevant to the human condition of CKD which has not been shown fully for species which are less sentient . We use terminal anesthesia for some of our planned studies in order to refine our studies and decrease animal distress/suffering.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have refined the models we use over previous PPL projects. We now routinely group house post- surgery which has improved animal post-surgery condition scores. We have increased analgesia post surgery up to 72hrs when required which has also improved animal condition scores. We use animal condition scoring sheets for all animals who have had renal disease induced regardless if asymptomatic or not. These sheets document weight, body condition, clinical signs of any discomfort and distress as well as including measures of renal function were appropriate. This ensures that all animals are carefully monitored and ensures we maintain humane endpoints for all animals used.

For certain procedures for example blood pressure monitoring we train the animals so they are used for the procedure prior to taking measurements. This allows the animals to get

used to the handling and measuring apparatus reducing distress when measurements are made.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Where possible we used published best practice guidance in our experiments. For example for our diabetes models we used the guidance of the Diabetic Complications Consortium (DiaComp) publications. For our refined version of the subtotal nephrectomy we published the model ourselves and have a set standing operating procedure (SOP) which is followed by those training and using the model. Similarly for the other models we have written SOPs which we use for training purposes and to ensure best practice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To ensure that we stay informed about advances in 3Rs we will attend our University 3R's day held annually and the PIL refreshers course which updates on important new advances for example tube handling. These new advances will then be utilised during this project. We will also make use of the NC3Rs website news to ensure we are up to date.

125. How do Brains become Epileptic?

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

epilepsy, seizures, children with epilepsy, brain circuits, antiepileptic drugs

Animal types	Life stages
Rats	juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to understand how epileptic seizure activity in our brains makes it more likely that seizures will happen. We are asking how a first seizure might cause more seizures to follow.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Nobody knows how epilepsy becomes established in the brain, but recent work has shown us that brain circuits adapt themselves all the time to ensure that individual cells are neither too active nor underactive. We will use this new understanding to investigate how seizures recruit in-built mechanisms of adaptation to establish maladapted, epileptic circuits.

What outputs do you think you will see at the end of this project?

We plan to measure how many seizures an epileptic rat has and so determine the burden of their disease. Some may have lots of seizures, others will have very few. After assigning animals into groups, we will investigate the changes in the brain that are associated with mild epilepsy, severe epilepsy, and epilepsy that is either hard to treat or is very responsive to drugs. Armed with this information, we will perform a large amount of tests to see how the genes and proteins and brain cells differ between the groups so we can identify why some brains have more seizures than others. So that we can relate this to children with epilepsy, we will perform the same tests on brain samples taken from children with epilepsy who have surgery to take out the epileptic parts of their brains. Secondly, we are working to understand how the immune system contributes to epilepsy, and identifying how we can use drugs to prevent the body from fighting itself to cause inflammation of the brain that leads to more seizures. Finally, we will test new drugs on both epileptic animal brain tissue and tissue from children, to see if we can identify a new drug to treat the hardest-to-treat epilepsies. Our outputs will include publications, so that other scientists can make use of our work, and a data repository, so that scientists around the world can use our data to generate new insights.

Who or what will benefit from these outputs, and how?

Children with hard to treat epilepsy will benefit, because we are working with a drug company that is trying to make new drugs to treat epilepsy. We have already made a new drug which is taken from cannabis plants, and this is working well in the most severe epilepsies. We are now looking for drugs that alter the process of establishing epilepsy itself. We call these drugs 'disease modifying' because they don't just stop the symptoms, they treat or prevent the reasons for the symptoms themselves.

Our previous work took 9 years to get to a drug treatment, we would hope to have a similar timescale for a new drug this time. The increases in our understanding will, however, come along earlier, in perhaps 3-5 years.

How will you look to maximise the outputs of this work?

We are collaborating with Pharma (drug) company, who are also funding some of the work. The other half of the support is from the Medical Research Council and between them, they have given us more than £2M pounds towards our project. We are also collaborating with colleagues in a British University, and a University in Berlin who help us to identify and copy immune antibodies in epileptic brains. This part of the project is funded

by another £1.5M and we have already published several papers, with many more to come. Importantly, we have developed several new models of epilepsy (with NC3Rs funding), autoimmune encephalitis and the use of human tissue in brain studies such as this. All of these new models have been published and are being used by other investigators around the world.

As noted above, our outputs will include publications, so that other scientists can make use of our work, and a data repository, so that scientists around the world can use our data to generate new insights.

We meet with patient groups to disseminate our findings, make videos and podcasts of our work, and are at present setting up a website for our research projects.

Species and numbers of animals expected to be used

- Rats: 800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use rats because they are the lowest organism that models the disease with good translation to humans, and we will directly test translatability using human brain experiments. We will use young rats, because like humans, they are more susceptible to epilepsy. We will use human antibodies taken from children with epilepsy to make epilepsy in young rats brains, so ensuring that the models are as close to human disease as possible.

Typically, what will be done to an animal used in your project?

We use two approaches: the first is to generate epileptic seizures or other altered brain activity using drugs and then to record them over time using electrodes that send brain waves from implanted electrodes over to a computer for analysis. In this approach, electrodes are implanted into the brain surgically, under general anaesthesia and a week or so later, drugs are injected subcutaneously between the shoulder blades, seizures are monitored and treatments administered via the drinking water.

In the second approach, we use surgery to implant tiny pumps which deliver antibodies to the brain to induce epilepsies that directly mimic the syndromes in children from whom we have taken the antibodies. Again, we record the brain waves and treat the animals with drugs and make measurements to understand what has changed.

We will also make use of animals that are genetically modified so that they cannot generate inflammation in the brain, this will allow us to explore the role of inflammation in epilepsy.

What are the expected impacts and/or adverse effects for the animals during your project?

Epilepsy is not normally painful, either for humans or animals. The main impacts will come from having brain surgery, which involves inserting a very thin wire into the brain and attaching it to a radio telemetry device and a battery pack that is placed between the shoulders. Surgical discomfort will last for a 24-48 hours after operation and we will administer painkillers for this. Our model is very similar to human epilepsy, and so some rats may take many months to develop epilepsy and be epileptic for several months while we record their brain activity. The long time course, variability between animals and slow progression make the model as realistic as possible, but mean that in some animals seizures may persist for several months but never more than 12 months.

Other potential harms include post-operative would infection, but this is rare. With any epilepsy procedure using drugs to cause seizures, weight loss occurs because seizures cause a lot of muscle activity. Our current limit is 20%, but since all animals recover extremely well in our hands, and the weight loss is related to aerobic exercise of muscles and not to illness or loss of appetite, we would suggest 25% as a limit.

Our genetically altered animals do not appear to suffer any harms from their loss of a specific subtype of brain cell. This cell normally fights brain infections and so there might be an extra risk should animals become infected during surgery to implant electrodes. We will take extra precautions to ensure aseptic technique with these rats.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The epilepsy models are mostly of moderate severity, because of our numerous (previous and current, all published) refinements. Mortality is around 1% from induction of epilepsy, and this mostly relates to uncontrolled spread of seizures to the brainstem in a few highly sensitive animals. Our models are pioneering and unique in such low mortality and the fact that the most sensitive animals do not die means that instead of studying 'survivors' of an unrefined procedure who are by definition the least sensitive animals, we capture a spectrum of disease severity that resembles that in children, who may have 1 seizure a week or as many as 200 per day. About 60% of our animals may experience moderate severity, and the rest of the work will be mild. 1% of animals may suffer severe severity through being highly/fatally sensitive to seizure induction.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The fundamental basis of the work requires intact brains. As an example, if we apply the main convulsant drug used in the project to a slice of isolated hippocampus nothing happens. This is because the convulsant action requires communication between the thalamus and the hippocampus, and it means that whole animals are essential to the successful establishment of epilepsy. Similarly, we are looking to find drug treatments and biomarkers for disease severity, and for this we need not just brain tissue, but tissue from the liver and other organs. Again this means that a whole animal approach is essential so that we can obtain a wealth of genomics, proteomics and lipidomics data.

Which non-animal alternatives did you consider for use in this project?

We make parallel experiments in cell cultures from human brain tissue taken from epileptic children. We use acute (non-culture) brain tissue as well, and we make this our 'gold-standard' for comparison to our animal work. Most of our work has parallel investigation in human brain tissue and we regularly compare results across models.

Why were they not suitable?

Non-animal tissue is eminently suitable, but there isn't enough of it available to run the project entirely without rodents. We have access to about 40 patients per year for the human work, and we are working hard to make this into a resource that can be shared with colleagues round the UK so that animal work can be validated, reduced and refined wherever possible.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We need to record from animals for many weeks or months after surgery, and so the number is relatively low compared to project where investigations are more acute. We base our numbers on the amount of spaces in our telemetry unit, the cost and availability of electrodes and the requirements for statistical rigour. Our previous work has informed out estimate and we have assumed we will secure follow-on funding in addition to the £3M we currently have secured.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We performed calculations based on how likely it is that animals will experience more or less severe disease, and so be stratifiable into groups, and on the variance between different data sets. For example, when measuring neuronal network oscillations, the variance is sufficiently high as to require as many as n=20 animals per group, but when measuring expression of specific genes, the numbers are often much lower (n=7). We have a a research group been previously funded by NC3Rs to refine our models, and we have consistently made new refinements within the last decade.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We are sharing tissue with a Pharma company who are providing all of the 'omics analysis. Omics is an umbrella term for the generation of large datasets which effectively provide a map of something. For example, a genomics study might provide a map of which genes are expressed under certain conditions and a proteomic one would reveal which proteins are made at any one time in a specific tissue. There are many variations of these sorts of datasets, depending on the questions being asked.

We are also sharing tissue with collaborators at a UK University such that a single animal will provide tissue to our establishment, our partner University, and the Pharma company, and most importantly, changes will be correlated across the sites so that we can link electrophysiology to molecular analysis of receptor function, expression and trafficking (our partner University) and thence to brain and other organ 'omics (Pharma).

Secondly but no less importantly, we also optimise our brain slice preparation, using a range of techniques and interventions developed over the last 30 years in the main laboratory, so that maximum data is obtained from acute electrophysiology because our slices are amongst the best in the world today.

Lastly, we will breed all our animals at our establishment, allowing us to optimise numbers as the project develops and prevent unnecessary culling.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use the RISE epilepsy model developed in my laboratory using NC3Rs funding. This model has 1% mortality compaed to the 40-80% of other models around the world. The model tightly controls seizure induction, duration and cessation, and captures the full range of responses from mild disease through to the most severe epilepsies. Other models kill many of the most susceptible animals, such that investigators are, by definition, working on the least seizure susceptible brains. The other epilepsy models have also been developed in my laboratory, using antibody infusion in juvenile rats chosen to be at the precise point of maximum seizure sensitivity and these animals show seizures where many labs are unable to demonstrate more than abnormal activity. In addition to the RISE model, we have developed models of anti-NMDAr encephalitis, anti-GABAr encephalitis, anti-LGi1 encephalitis and FIRES epilepsy.

All of the work to date aims to use models we have developed to show the lowest mortality, and hence the least distress. All of our surgeries now use a modified approach, developed in our lab, that minimises the surgical process, reducing incisions, recovery time and maximising the robustness of electrode caps. We strive constantly for better approaches and outcomes.

Why can’t you use animals that are less sentient?

Earlier life-stages would involve taking animals away from lactating mothers, and would also mean that electrode and battery/transmitter implants would be too bulky relative to body size. Neither of these is desirable. Less sentient species, such as invertebrates, do not possess similar enough brain structures to be comparable to human epilepsy.

Mice would be a potentially less sentient species than rats for this project, however, mice cannot be wirelessly recorded in the same way due to their small body size relative to the implanted transmitter, and their genetic background is unsuitable for the experiments aimed at investigating inflammation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have refined our surgery approach to reduce the size of incisions, reduce the number of incisions needed for telemetry from 2 to 1, and to prevent adverse effects such as the

electrode cap falling off (by development of a new gluing process). One of our protocols calls for repeated intraperitoneal injections and we have now refined this so that the drug can be delivered solely in drinking water.

As mentioned above, we have refined our surgical approach at the level of operative technique, we have refined our practice for skull-capping and we have altered our epilepsy models to minimise mortality. We continue to look for opportunities to further refine our work and always use post-operative scoring sheets, pain management, enhanced nutrition and care after procedures. One arm of our work explores the relationship between schizophrenia and epilepsy using sub-chronic PCP dosing.

Normally, scPCP models use intraperitoneal injection, twice daily, for 8 days, followed by similar for clozapine injection. As rats become psychotic, injection is more and more stressful and difficult even in the best hands. This profound stress must surely confound experimental data. We have had great success in refining this model such that drugs are administered via drinking water, after some training of rats to enjoy Ribena. We aim to publish this a significant refinement and this demonstrated our ongoing commitment to the 3Rs, our culture of care and my belief that the best science goes hand in hand with the best animal treatment.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We abide by the ARRIVE guidlines, ASPA and LASA guidelines and we perform regular Harm-Benefit analyses based on elements flagged up by students, post-docs and others in the lab. No technique is fixed to the point where we can't consider a refinement, and no lab member is without a voice when it comes to animal welfare. Our technical team are clear that they can flag issues with academics at any time and there is regular, fruitful and authentic dialogue between all parties.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am the ethics advisor to PLoS ONE journal, I have sat on government committees and I currently act as a Home Office advisor to my University as well as running the animal facility. I sit on several ethics committees with in the University and I chair an animals in science committee. I also have regular meetings with our NVS, and attend various forums provided by ASRU for office holders under the 1986 Act. This means I am in a good position to keep up to date on ethics. We also have a NIO, who keeps up with latest news from ASRU and NC3Rs. I have been funded by NC3Rs and developed the current epilepsy model using that grant, hence I keep up to date with NC3Rs as I seek further opportunities to improve animal science.

126. Enzymes and Other Pathway Components Linked to Neurodegeneration

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims: 0

Key words

Parkinson's disease, Neurodegeneration

Animal types	Life stages
Mice	embryo, adult, pregnant, aged, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our research is aimed at understanding how serious neurological disorders such as Parkinson's disease (PD) arise in humans. We aim to develop a deeper understanding of the causes of PD and exploit this knowledge to develop better ways to diagnose and treat this disease in the future.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Parkinson’s disease is the fastest growing neurodegenerative disease in the world, with currently afflicts ~150,000 people in the UK and up to 10 million people worldwide. Currently available medications can’t slow or stop the progression of the disease and are aimed at alleviating symptoms. Our goal is to undertake high quality research that contributes to improved understanding of the causes of PD, which we hope will lead to the identification of new therapeutic targets as well as better strategies to diagnose Parkinson’s disease. The focus of our current research is on Parkinson’s disease but in the future we might wish to exploit our expertise to undertake analogous work to better understand other neurodegenerative disorders such as amyotrophic lateral sclerosis.

What outputs do you think you will see at the end of this project?

The primary output from this project will be new information on the mechanisms underlying Parkinson's disease. This will be translated into peer-reviewed publications. My previous project licence has generated 11 papers published in high impact factor journals as a direct result of animal work and I would expect at least a similar level of publications for the next 5 years. Our research is also likely to have significant translational impact. A main focus of our research is a gene called Leucin Rich Repeat Kinase 2 (LRRK2) that has been implicated in Parkinson's disease. Research in the LRRK2 field has reached a critical juncture. Clinical trials are on-going and there is increased urgency to better understand how LRRK2 is linked to Parkinson’s disease. The mouse models we are studying will significantly help with this. We hope that our future work will reveal new potential therapeutic targets and better ways to assess the activity of LRRK2 in human samples. We hope our work will also lead to new tests that would identify Parkinson’s patients whose disease is driven by LRRK2 and would therefore be most likely to benefit from drugs that target LRRK2.

Who or what will benefit from these outputs, and how?

Our goal is to develop a better understanding of genes (such as LRRK2) that have been implicated in Parkinson’s disease in humans. Our research could help develop new diagnostic tests to better identify Parkinson’s patients whose disease is driven by LRRK2. Drug companies have developed potential new treatments that target LRRK2 and our work could help identify patients who might benefit most from these new treatments. This is an example of how patients may benefit from our research soon (1- 2 years).

Our recent work with mice has revealed that PD patients with mutations in another gene implicated in Parkinson's disease might also benefit from LRRK2 targeted therapies that are currently in clinical trials. We understand that this is now being considered by one of the companies working in this area. This is another example of how patients may benefit from our research soon (1-2 years).

In the last few years, our work with mice has helped demonstrate that another gene is also linked to LRRK2. These results have enabled us to obtain funding to initiate a project to

identify compounds that target this gene. If this project is successful, it could benefit patients in the long term (10-20 years).

Our previous animal studies have led to many collaborations with other researchers, clinicians and pharmaceutical companies who have further exploited our animal models and cells derived from these in their own analysis to drive the research field forward. Over the last 5 years we have shared over 20 mouse models, as well as tissue samples and cells derived from these, with other research laboratories worldwide to help support Parkinson's disease research.

How will you look to maximise the outputs of this work?

We're committed to open access policies and open sharing of all outputs of our research. We're part of a collaborative network that currently consists of over 800 researchers worldwide (Principal Investigators, as well as Research Assistants and Students), with whom we regularly share the outputs of our work including unpublished data and unsuccessful approaches. We also share the research tools that we develop with academic collaborators and with industry, allowing others to directly benefit from, and exploit the research that we undertake. We provide our mouse models and cells derived from these to numerous other laboratories worldwide.

In addition to the direct scientific benefits, we perform significant patient and public outreach. For example, we work with Parkinson's charities in the UK to provide a forum for patient talks to explain the science that we perform and how this adds to the knowledge of their disease. The close scientific links between our group and the clinics at the local hospital have led to Parkinson's patients coming to our laboratory from nationwide to discuss and better understand their condition and the research that we are undertaking. We have also held virtual, web-based calls with patients based in other countries.

Species and numbers of animals expected to be used

- Mice: 28,200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We make extensive use of biochemical, cellular, gene editing and increasingly computer modelling systems to develop a better understanding of Parkinson's disease. This greatly reduces the need for us to undertake much of our mouse work as we can now study how mutations in disease genes impact cells without the need to generate a genetically altered mouse model. We now routinely deploy techniques to generate genetically altered cell lines that allow us to answer several questions that previously could only be addressed in

mice. Nevertheless, it is still essential that we can rigorously establish the impact that disease genes have on complex tissue physiology including brain, kidney, lungs and spleen. Work performed in mice also enable us to assess the effects of age, that is a key risk factor in Parkinson's. It is currently impossible to adequately assess ageing in cells grown in the lab. Another issue is that Parkinson's disease involves the interaction between many brain cells, as well as the interaction of the brain with the gut, and the immune system. These interactions cannot be easily assessed using tests on cell lines. For these reasons, mouse work will continue to play an important part for our research in the area of Parkinson's disease as this provides us with much greater insight into how mutations in genes linked to Parkinson's impact on whole animal biology and disease processes.

We are currently investing significant effort in developing genome editing technology to better generate human cell lines that harbour Parkinson's mutations. These engineered cells will further reduce the number of mouse studies and models that we will need in the future. However, it will still be very difficult to introduce multiple mutations in different genes which we need to do when studying the causes underlying Parkinson's processes. For these experiments that investigate interactions between multiple genes, we will still need to generate mouse models.

We conduct many of our scientific investigations on tissues and cells derived from adult animals. On rare occasion we conduct our investigations in pups or embryos. Specific instances where this is necessary are:

1) Isolation of neurons from the brain. These cells can typically only be obtained from one- or two-day old pups and are then grown and analysed in the lab. Unfortunately, these cells currently cannot be grown and propagated in long term in the lab. Therefore fresh cells are required for each batch of experiments. Parkinson's results from the loss of neurons that produce dopamine. Use of dopamine neurones isolated from mice would enable us to study how diverse Parkinson's genes impact physiology and affect dopamine production. It would also enable us to test whether therapies that target LRRK2 would boost the survival of neurons.

2) On occasion we isolate cells such as fibroblasts that are derived from mouse embryos. These cells can be propagated in culture in the laboratory and therefore we only need to generate these cells once. These experiments enable us to better investigate the impact that mutations have. These cells can also be provided to other laboratories all over the world to help researchers with their work on Parkinson's biology. Therefore these cells can have broad and major impact.

Occasionally we may employ the use of mouse models termed "reporter lines" (where there is a specific "marker" we can detect). These allow us to accurately pinpoint specific cell populations for use in downstream scientific investigations.

Most of our research is undertaken in tissues derived from mice typically maintained for up to 16 weeks that show no detectable clinical signs. These mice are killed by approved methods, so the animals have no harmful procedures done whilst they are alive. For a few mice, we may need to look at subtle neurodegenerative changes that might be age-dependent. These mice might need to be maintained for up to 2 years. In these cases, we shall monitor animals closely and will euthanise any that start displaying welfare problems. Careful post-mortem analysis will be performed on the tissues from these mice to understand whether these animals display signs of neurodegeneration.

Typically, what will be done to an animal used in your project?

Wherever possible, we conduct our research on tissues and cells derived post mortem from the animals used in our project, with no intervention being necessary in live animals. Usually, the animals are killed by a Schedule 1 (approved) method, but sometimes a greater scientific value can be achieved by adopting another method under deep and irreversible anaesthesia (no more than 25% of the animals will be killed by one of these humane, but non-Schedule 1, methods). Specific instances when this is necessary are for particular types of tissue preparation and if we need to take cerebrospinal fluid (the fluid that baths the brain and spinal cord) for analysis. For harvesting some types of nervous tissue from very young pups (1-2 days old) we may use decapitation in animals that are not under anaesthesia. This has been shown to be a quick and humane way to kill the animals and causes no welfare issues.

To characterise the effects of specific genetic alternations we may also perform basic studies to look at the effects of the changes on the phenotype (an individual's observable traits) in a limited number of animals. This may involve minimally invasive procedures such a taking a small blood sample for analysis or observational tests such as seeing if an animal has poorer motor skills by testing how good it is at balancing, etc. In some cases, we may need to use slightly more invasive investigations of metabolic function by giving a non-toxic compound and seeing if the genetic changes alter how the compound is absorbed or metabolised (pharmacokinetic studies). To test the effects of these compounds (protein kinase inhibiting or activating agents) we dose the animal with a small volume given by the least invasive route possible. Food and water are the preferred routes, but where these are not possible, we may need to gavage (dose by stomach tube) or inject compounds. In many of the small number of studies where we use these compounds, a single dose is sufficient to provide the response we require. We may take blood samples from dosed animals at intervals (of the smallest feasible volume), in order to measure metabolic responses to pharmacological interventions or to measure levels of the compounds dosed. After animals are humanely killed we do extensive post mortem analysis.

The vast majority of animals will be kept in cages for less than one year (our aim is 6 months). In some cases we may need to maintain animals for a longer period of time (up

to 2 years) for extended metabolic or neurological phenotyping that might be age-dependent, such as subtle neurodegenerative changes.

To fully appreciate the complexity of the central nervous system there is a need for appropriate behavioural testing. Parkinson's disease and other neurodegenerative conditions are often associated with motor deficits. However, associated non-motor symptoms can manifest themselves years and sometimes decades before the onset the motor phenotype in people. Non-motor symptoms include hyposmia (loss or reduced ability to smell odours), sleep abnormalities, gastrointestinal disturbances, anxiety, depression, dysfunction of the autonomic (non-voluntary) nervous system, and impaired cognition. Behavioural testing for traits in mice that mirror these aspects of disease in people is highly specialised and we believe that this is best performed by researchers who are experts in this area. We have strong collaborative links with other laboratories who have the relevant skills and equipment. Therefore, should a need arise for such testing, our strategy would be to collaborate with these groups and transfer animals from the relevant breeding colony to other facilities to perform such behavioural tests. Animals that are to be transported are checked by the vet or experienced animal technician to make sure that they are well and that their welfare will not be adversely affected by transport. Such testing is expected to add to our understanding of the underlying disease mechanism, earlier diagnosis and possibly the development and testing of new therapeutics.

What are the expected impacts and/or adverse effects for the animals during your project?

A detailed description of the expected adverse effects is specified under each Protocol.

In our experience, mouse models of Parkinson's disease exhibit very few signs of adverse welfare, though tissues analysed after the animals have been humanely killed demonstrate informative molecular changes. We do not require animals, if they do show any signs, to progress beyond early signs of disease. Animals may be administered chemicals that are believed to be potential new medicines, to measure their effects on the cellular networks that we study and on the development of any outward signs of disease. These chemicals are not, in themselves, expected to cause any harm.

Animals undergoing procedures (e.g. injection, gavage, etc) are not expected to experience anything other than minor, transient discomfort. Any animals showing signs of suffering that are greater than minor or transient, or in any way compromises normal behaviour, will be culled via a schedule 1 method. Some mild or moderate signs of adverse, age-related effects may be expected in animals that are maintained into middle to late adulthood and may manifest themselves initially as deterioration in general wellbeing.

Although many of the lines we use might be expected to have changes in neurological function, we do not expect to have animals that demonstrate more than subtle changes in phenotype in most instances. In cases where animals are expected to be phenotypic, the

relevant end-points will be described and a harm benefit assessment of their use to consider the likely scientific benefits against the potential harms performed locally via a system approved by the local Welfare and Ethics Committee (currently performed by consideration of the Named Veterinary Surgeon of relevant study plans).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Two from three protocols in our Project Licence have a moderate severity limit, while one protocol has a mild severity limit.

Protocol 1 - Breeding and maintenance of GA mice (MILD). We expect a very small proportion of the animals in this category to experience a MILD severity limit. The phenotype(s) are expected to be sub- threshold and genotyping will generally be undertaken using surplus material from ear notching for identification. There may be instances where a mouse may experience a MILD severity, such as rebiopsy for genotyping purposes. Mice produced and maintained under this protocol will be used as future breeders for colony maintenance and tissue analysis.

Protocol 2 - Investigation of Gene and Protein Expression (MODERATE). Mice transferred to this category will undergo regulated procedures but we would expect under 50% to experience a MODERATE severity limit. Many will undergo only one regulated procedure, so these mice will experience minor and transient discomfort, but some mice may be subjected to multiple steps in this protocol. These steps individually may be described as minor and transient but cumulative harms must be considered over the lifetime of an animal. There may be some mice who will experience a severity limit of NON RECOVERY if tissues are collected under terminal anaesthesia.

Protocol 3 - Compound exposure in utero, pre and post weaning (MODERATE). Up to 75% of animals transferred to this protocol may experience a MODERATE severity limit. Parental females will most likely undergo multiple procedures considered as having a MILD severity limit, so cumulative effects can dictate a severity limit of MODERATE be assigned. Male breeders will experience a MILD severity limit where compound is administered via diet to female partners. Work will be staged on this protocol beginning with embryos, then moving to neonates, only if the embryo data dictates that neonates are required for the experimental aims. Neonate genotypes used in the experimental design do present a harmful phenotype, but these must be used to ascertain if the compound administered restricts the adverse effects, so we could reasonably assume that up to 75% of the animals on this protocol could experience a MODERATE severity limit. This percentage takes into account females subjected to multiple procedures and pups exhibiting and adverse effects.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To undertake our research plans effectively it is sometimes essential to make a mouse model that mimics disease-causing mutations found in humans. These animals enable us to rigorously assess the impact that gene mutations have on complex tissue physiology including brain, kidney, lungs and spleen and how these lead to disease in humans. We therefore require young and aged genetically altered animals as a source of precisely altered, but otherwise normal tissues and cells, and to allow measurement of biomarkers in fluids such as blood that is not achievable using cell line models.

The animals and cell lines derived from mouse models also greatly support medical research that is being undertaken in this area by our lab as well as others who we collaborate with and provide reagents to. They enable us to validate research findings made using non-animal approaches, such as our cell line work, for example, proving that kinase-A does indeed phosphorylate substrate-B in a mouse tissue such as brain. These results provide a robust framework of knowledge of how these signalling pathways operate and are organised. This information is crucially important for persuading pharmaceutical companies to invest in this therapeutic area. Moreover, if mutations result in a disease- like phenotype, the animals and cell lines derived from these could serve in the future as a preclinical therapeutic model, in which new inhibitors undergoing preclinical evaluation can be assessed

Furthermore, in many disease areas such as Parkinson’s it is not clear what the best cell line is to study the molecular mechanisms underlying this condition and there are caveats with any type of cell line selected. There are debates amongst experts in the field on how well neurons derived from differentiated embryonic stem cells mimic adult neurons found in human adult brains.

The mouse models and cell lines that are derived from them (i.e. fibroblasts and primary neurones) that we use for the vast majority of our work are also provided to other laboratories including pharmaceutical companies with different expertise. This supports and greatly extends the impact of our research much beyond the original scope of research undertaken in our laboratory.

For many projects, we would now envisage no longer generating mouse models and relying on data obtained from CRISPR/CAS9 genome edited cells. This can be useful for validating “hits” that we obtain from various genetic and mass spectrometry screens.

However, for the most important new findings that we make that we are able to validate first in cell line models, we envisage that we would still need to generate mouse models as cultured cells are not always good analogues of the brain and other tissues. For these highest priority projects, mouse work will continue to play an important part for our research. Overall such studies provide greater insight into how mutations in the genes of interest impact on whole animal biology and how these may ultimately be linked to diseases such as Parkinson's.

Our work on occasions results in us combining knock-in or knock-out mouse strains to study the interactions that arise between two genes in vivo. These multiple mutant strains have the potential to provide valuable information on how combinations of genes relate to Parkinson's disease.

Which non-animal alternatives did you consider for use in this project?

We already make extensive use of in vitro systems to probe the molecular properties and substrate specificities of protein kinases and phosphatases and other enzymes. The new genome editing CRISPR/CAS9 technology that has been developed in recent years allows us to rapidly generate knock-out as well as knock-in mutations in any cell line. This replaces the need for us to undertake a significant proportion of our mouse work as we can now study how mutations impact on signalling pathways in cell lines without the need to generate a mutant mouse. When we study a new protein, or a new mutation in a protein of interest, normally the first step is to generate knock-out and/or knock-in cell line using the CRISPR/Cas9 technology. If the data obtained using this system robustly demonstrates a major role for the protein/mutation in the cellular pathway we're studying, we would consider generating a genetically altered mouse model. Many mutations we generate in cell lines would be expected not to result in a major or any relevant phenotype and in these cases no mouse studies would need to be undertaken on these components.

We also use frequently attempt to study the pathways of interest in cells such as fibroblasts, neutrophils, or monocytes derived from human patients bearing mutations in the component of interest. These are sourced through national and international collaborative links with leading clinical researchers. If such material is accessible, we would expect to perform this analysis prior generating mouse models.

Why were they not suitable?

For some aspects of our work, particularly in the Parkinson's disease area, cultured cells are not always good analogues of the brain tissues/neurons. In addition to this, currently available genome editing technology does not readily allow the generation of cell lines harbouring multiple knock-in mutations in different genes.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of mice is based on my previous PPL which covered a broadly similar type of work as the new licence and where the vast majority of animals are used within the breeding protocol, with subsequent scientific investigations post mortem. We regularly review our breeding strategies to ensure that they are appropriate and use as few animals as possible. Strategies are tailored to individual lines and we prioritise animal welfare such that some lines have to be bred in a way that we use more animals to achieve the genotypes that we want, but that avoids producing ill animals or animals that might otherwise have breeding problems, for example we observed that heterozygous females for one line were smaller than expected, which resulted in issues when these animals were giving birth. As a result, in consultation with the NVS we adopted a change in the breeding of these lines for which we now run two colonies side by side for mating (to avoid generating female breeders that are heterozygous for the relevant gene).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When designing a study we deploy the Experimental Design Assistant tools developed by the NC3Rs in order to design experiments that will give robust answers while not wasting animals. We carefully calculate the number of mice needed to obtain statistically significant data and ensure that we breed only these precisely these number of mice. We have also refined our technologies to reduce the number of mice required. For example, we have been recently undertaking immunoprecipitation of lysosomes from mouse tissues and analysing these for protein lipid and metabolite levels. Original technology required 3 animals for these analysis. We have optimised the method boosting sensitivity of our assays over the last year and we can now perform all analysis from a single animal reducing the number of animals required for this study by two thirds.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We manage our breeding colonies very carefully to ensure we breed only the number of animals required for analysis. Within our research Unit, we employ a full-time staff member who manages the mouse colonies ensuring efficient breeding and use of animals. For example, we use the same parents to generate experimental animals and to set up timed mattings to generate embryos for subsequent isolation of embryonic fibroblasts. We have an active cryo-preservation programme, so current breeding can be restricted to those lines being actively worked on.

For projects that involve new tests that have never been undertaken before, before performing a large experiment with many mice, we would always undertake a pilot experiment with the smallest number of mice to ensure that the experiments we are aiming to perform are feasible. This could include undertaking a pilot study aimed at assessing whether a protein(s) or measurement of interest can be accurately undertaken in amount of mouse tissue(s) available. Before administering a new compound to a larger cohort of animals, a pilot experiment with a small number of animals would be undertaken frequently testing various doses and formulation of compounds to ensure that we can confirm an optimal dose and mode of administration to maximally inhibit the biological pathway being assessed.

Tissues collected from same set of animals would normally be used for multiple downstream analyses and kept frozen in the freezer in case this can be also used for future studies. In addition, we frequently share the mouse tissues and cells generated from our animal studies with other academic collaborators and with industry, allowing others to benefit and exploit the research that we undertake.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the chosen species for all experiments, primarily because of the ease with which it can be genetically altered and the molecular similarities between their nervous systems and our own which make them the ideal species in which to investigate the basic mechanisms and organisation of signalling pathways and how this might link to neurological disease. A contemporary list of GA lines being bred and maintained under the authority of this licence will be held.

Our studies will not require animals to exhibit signs of advanced disease, indeed the majority of the animals will live apparently normal lives and will be killed humanely before tissues are harvested for detailed laboratory studies. All mice will be very carefully monitored to minimise welfare costs including monitoring signs of reduced weight loss, neglect of grooming, reduced ambulation, early signs of movement impairment and resistance to passive movements.

We use post mortem analysis to perform as many of our studies as possible so that few of our animals have any invasive procedure while they are alive. Where we do need to

perform studies in the live animals, we use minimally invasive methods (observational behavioural testing, small numbers of blood samples and/ or one or more small doses of non-toxic compounds by the most refined routes).

Why can't you use animals that are less sentient?

Due to the nature of our work that involves understanding the molecular mechanisms and organisation of signalling pathways involved in Parkinson's Disease, a mammalian model is much preferred as we believe this will be closer to the human system. Employing a mouse system, the data we obtain will be more relevant for pharmaceutical companies to assess the physiological relevance of our data for their drug discovery efforts. A major goal of our work is to mimic the Parkinson's causing mutations in humans and study the impact that these variants have and the mouse model is the most suited animal model for the research that we are proposing in terms of ease and cost of generating and maintaining the animals. As Parkinson's is an adult-onset disease, we need to be able to use adult animals to be able to fully investigate the effects of changes in function in the pathways of interest. Most work would be undertaken in mice of under 6 months of age, but on occasion it is important that we can perform experiments in older animals of up to 2 years of age.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All procedures to be performed on mice will be described in specific study plans, to be discussed and lodged with the animal facility and NVS and will be made available to the Home Office inspector on their visits.

We may administer very well characterised compounds (such as drugs targeting LRRK2 that are now undergoing clinical trials) to examine their effects on specific pathways that we are studying. We will administer these by the least invasive route. Combining the compounds with food and water for long- term administration (2-28 days) is our preferred administration route. We currently administer a LRRK2 inhibitor for such long-term studies in chow and we find this approach to work extremely well and avoids twice daily injections or gavage treatments. Where this approach is not possible, for example for a compound that we have a limited supply of, or is not sufficiently stable or tastes too bad to add to food/water, we may need to administer this by gavage or injection. In the majority of our studies, a single dose of compound is sufficient to provide the response we require and in this case, injection is the preferred route. Before administering compounds to a larger cohort of animals for the first time, pilot experiments with a small number of mice may be undertaken with varying doses and formulation of drugs to ensure that the drugs administered are having the expected effects on signalling pathways. These studies will also ensure that the drugs are well tolerated by the mice and not cause harmful effects. Animals will be monitored closely following all procedures until normal behaviour resumes.

Any experimental work which involves the administration of compounds to embryos and pups, will be subject to a staging process. The compound will be administered, in diet, to females just prior to, and during, pregnancy; then embryo collections will be carried out a short time after the mated females show signs of pregnancy. Any females showing no signs of pregnancy will be culled to ensure that no embryos, whose age cannot be accurately determined, are allowed to develop. If the full range of embryo genotypes are detected, and the genotypes are outwardly healthy, only then will embryos be collected at increasingly older ages with each sequential experiment. If the compound appears to 'rescue' the embryos from the potentially harmful phenotype, discussions with the NVS will then take place to ascertain whether the work can then move onto a phase where pups are produced. The pup experiments will be subject to a similar staging process as used in the embryo experiments, whereby each sequential experiment using progressively older pups, will only be approved if the previous experiment produced pups not subject to harm. This refinement of the experimental design process ensures that the risk of pups being born with a harmful phenotype is kept to the absolute minimum.

We may take blood samples at intervals (of the smallest feasible volume), in order to measure responses to administered agents. Animals may be warmed gently (in a warming box or on a heating pad, or by immersing the tail in lukewarm water) to facilitate the collection of blood samples with minimal stress.

All animals that are aged will undergo weekly weighing and body condition scoring to monitor general well-being from the age of 6 months. This will be carried out by a trained and competent person, who is independent from the ongoing study, to reduce any bias.

In cases where animals are expected to display an overt phenotype, the relevant end-points will be described in detail. We will also undertake a harm benefit assessment of their use to consider the likely scientific benefits against the potential harms performed locally via a system approved by the local AWERB (currently performed by consideration of the NVS of relevant study plans). For phenotypic animals a GA passport will also be considered by an appropriate NACWO involved in the animal care. The contemporary list will contain information on the expected adverse welfare effects of the genetic alterations and the steps that will be taken to mitigate them. To further mitigate adverse phenotypes, we would also consider generating more refined mouse model in which the genetic change is limited to specific tissues such as the brain rather than occurring in the whole animal.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidelines published by the NC3Rs and LASA.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We work closely with the NVS and NAWCOs, as well as the staff running the animal facilities, to ensure that we're always up to date with any advances in the 3Rs and these are implemented effectively. Furthermore, all PPL & PIL holders in our Institution are required to complete Continuing Professional Development via relevant training mandated by the AWERB, including local refresher training and training by recognised providers such as the online training modules on the Research Animal Training website.

127. Interrogation and Identification of Existing and New Targets for the Treatment of Chronic Pain

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Pain, Anxiety, Arthritis, Ageing, Analgesics

Animal types	Life stages
Rats	neonate, juvenile, adult, aged
Mice	adult, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Aim: To advance understanding of the molecular, cellular, and pharmacological mechanisms underlying chronic pain responses, and how these differ from those in acute pain. To determine how these processes are altered across the life course, and in the presence of co-morbidities such as pre-existing anxiety, and to investigate why prior exposure to opioid analgesics worsens pain outcomes later in life. The new knowledge gained will then be used to identify treatments specifically targeting these mechanisms, to benefit people living with chronic pain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Everyone experiences pain following an injury, however this is normally short lasting in duration and protects you from further damage while the tissue repairs. There is evidence that in some diseases the pain people experience is not protective. This pain doesn't serve a protective purpose and stops peoples' normal everyday life. We know that the ways in which pain is generated, detected, and processed are different in different types of disease, at different times of life, and that the treatment of these types of pain requires different drugs to the ones we take every day for a minor acute pain. In some diseases such as arthritis, people start with short-lasting intermittent pain from the joint and over time this becomes more regular, more intense, and more debilitating pain. This change is not just because the disease in the joint is worsening, but also due to changes in the peripheral and central nervous system, we need to understand why this happens so that new treatments can be developed.

To address this problem we need to understand the mechanisms occurring at sub-cellular, cellular, and tissue levels that lead to chronic pain. We will achieve this by studying models of major clinical chronic pain problems in society, inflammatory pain, neuropathic pain, and musculoskeletal pain.

There is increasing evidence that people's experience of pain is shaped by events that take place in early life, interactions with emotions such as anxiety, and or neuropsychiatric conditions such as depression. For example, newly-born term and pre-term babies are exposed to a number of tests which activate the pain pathways, and it is now clear that these events may influence the experience of pain in adulthood. We will investigate how pain in early life effects the way the sensory nerves, spinal cord, and brain responds to pain in adulthood. It is clear from clinical data that mental health, in particular anxiety, modulates responses to acute and chronic pain. Why people with higher anxiety experience greater chronic pain is an important question which can be studied using animal models. At the same time, our work under this authority will be complemented by studies on clinical samples and populations as well as cell-based approaches to the study of pain.

What outputs do you think you will see at the end of this project?

We aim to publish comprehensive, high-quality, peer-reviewed papers, which we make available for open access.

Throughout the project, we will present preliminary findings in the form of poster presentations and talks at international conferences and through other invited presentations. In addition, once papers are completed for submission to peer-reviewed journals, we will place them on a pre-print server (eg bioRxiv). This will ensure that our findings are disseminated as swiftly as possible. This has the added benefit that the progress of our project can benefit from early feedback by peers.

We also aim to broaden the benefits of our research by including our findings in our teaching to undergraduate and postgraduate students, and by disseminating it to the

general public via our webpages and press releases, Brain Awareness Week events, Summer Schools for school students, Pint of Science events, articles in popular science journals, etc.

To maximise the impact of our research on the discovery of new pharmacological treatments, we collaborate with colleagues from the neuroscience drug discovery industry.

We also closely integrate our in vivo research in rodents with studies in patients suffering from chronic pain and will use similar behavioural and non-invasive imaging methods. This will help to maximise the translation of our findings to humans.

Who or what will benefit from these outputs, and how?

Chronic pain is a major worldwide clinical problem that impacts upon hundreds of millions of people every year. Pain arises following trauma and disease, as a consequence of medical interventions like surgery, or as a side effect of drug treatments. Acute (short lived) pain is a necessary survival mechanism alerting us to tissue damage. Chronic pain can negatively impact the lives of anyone, regardless of their age or sex. Chronic pain outlasts any tissue damage and has no beneficial purpose to the individual. It is more commonly seen in older people (e.g. in diseases such as osteoarthritis), however it is also seen in the youngest children born prematurely.

The outcomes of this research will have direct relevance to people living with chronic pain. The outcomes of our research will include advances in understanding of how experiencing pain in early life changes the way the central nervous system matures, and why this alters how individuals' experience pain throughout their lives. This new information will be used to identify new ways to prevent these changes and hopefully prevent long-term changes in pain responses in adulthood.

Pain can arise following inflammation, injury to sensory nerves (neuropathic pain), or from diseases such as osteoarthritis (OA). We will advance understanding of the processes underlying these types of pain, specifically what is similar versis different. This new knowledge will be used to identify new ways to treat these chronic pain states.

People in chronic pain are more likely to suffer from poor mental health, experiencing anxiety and depression. Our clinical research has shown that pre-existing anxiety worsens chronic knee pain 18months later. The mechanisms by which pre-existing anxiety can exacerbate chronic pain are poorly understood. Our research will provide new knowledge whihc will help explain why these interactions between pain and mental health occur, which will enable the future development of new treatments.

In many cases the only successful treatment for OA pain is a surgical replacement of the diseased joint. People wait long periods of time for this treatment and this period is increasing due to the backlog in operations following the pandemic. Whilst waiting for surgery, people are prescribed a diverse and increasingly strong set of pain medications,

escalating to the prescription of strong opioid analgesic drugs. Opioids like morphine are not generally effective for chronic pain but contrary to NICE guidelines, they are still prescribed. Sadly, the consequence of this prescribing is not just that people don't get pain relief, but that the opioids they are prescribed can worsen the pain and decrease the likelihood of successful pain relief following surgery. We will study the mechanisms underlying these detrimental effects of opioid treatment on chronic pain, with the aim to find treatments that can reverse the detrimental effects of opioid prescribing. The outcomes of this research will likely have huge long-term benefits for millions of people prescribed opioids every year. Overall, the translational research in this project licence will shed new light on these questions and directly influence the direction of clinical research to improve patients' lives long-term.

How will you look to maximise the outputs of this work?

To disseminate our findings to other academic, clinical and industry researchers across the world who focus on brain-behaviour relations in health and disease, we will publish our findings in high-quality, peer-reviewed research papers in high impact academic journals. In addition, we will disseminate our findings at national and international scientific meetings.

Our findings will also inform our teaching of undergraduate and postgraduate students and will be communicated to the general public as appropriate (via our webpages and press releases, Brain Awareness Week events, Summer Schools for school students, Pint of Science events, articles in popular science journals, etc.).

Species and numbers of animals expected to be used

- Mice: 700
- Rats: 1700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Experiments will be conducted in rats and mice, for the following reasons:

• Their brain, including the endogenous pain control systems and spinal cord, is similarly organised as in humans (Paxinos (ed.), 2004, The Rat Nervous System).

• Their brain is sufficiently large to enable selective manipulation and analysis of the sub-structures of interest.

- A wide range of behavioural tests are available to study the sensory and behavioural functions of interest (Wishaw & Kolb (eds.), 2005, The Behavior of the Laboratory Rat).

- Welfare demands can be well satisfied in captivity.

Typically, what will be done to an animal used in your project?

The typical experience will be that animals (rats and mice) will be:

Baseline behaviour; model induction,; behavioural testing until chronic pain established; therapeutic treatment; schedule 1 termination or transferred to Protocol 3.

Young animals will be aged from postnatal (P) day 7 through to young adult (>P40). Aged animals will typically range from 14 - 20 months.

An animal will only be subjected to one model of pain:

Models of arthritis pain: weight bearing, hindpaw withdrawal thresholds to mechanical stimuli (rat or mouse).

Inflammatory pain models: weight bearing, hindpaw withdrawal thresholds to mechanical stimuli and / or thermal stimuli (rat or mouse). Neonatal animals will only undergo these pain models.

Neuropathic pain models: hindpaw withdrawal thresholds to mechanical stimuli and / or thermal stimuli and cold (acetone) test (rat or mouse).

Animals are free to move away from the stimulus at any time. Tests are first carried out before any other experimental intervention to determine baseline responses for later comparison. Subsequent measurement of hypersensitivity using these parameters in secondary sites (sites not injured) is indicative of central sensitisation. To assess weight-distribution between the hind-paws, the animal is placed on two force transducers that measure the body mass being borne by each hindpaw.

Quadrupeds with unilateral injuries such as inflammation or arthritis favour the uninjured limb whilst standing. A shift in weight away from the injured limb, even when not observable visually, indicates hypersensitivity. Observations of general, non-evoked behaviours such as grid crossing (counting the number of times lines on a grid are crossed whilst walking), overall locomotor activity, rearing, and other exploratory behaviours may also be collected. Reductions in these behaviours are indicative of increased hypersensitivity, e.g. rearing onto hindlimbs will be reduced.

In some studies, neuropsychological status will be assessed via additional non-evoked behavioural tests. For example, anxiety status can be assessed via the open field assay or the elevated plus maze. Animals will be placed in the appropriate arena and allowed to

freely explore with resultant behaviour observed and scored (see table 1 for frequency of behavioural tests for each pain model).

Some of the models of pain are generated by the administration of compounds either into the hindpaw, or the joint or systemically, which act either directly to activate the sensory nerves, or local inflammatory cells or cause tissue damage. These compounds are distinct from the pharmacological tools used to investigate the neurobiological mechanisms underlying the pain states being modelled.

In some studies the effect of systemic administration of drugs and compounds will be determined. This will require single or repeated dosing of the animals following establishment of baseline behaviours.

Drugs may be applied only before, only after, or both before and after, establishment of models of chronic pain. The selection of drugs and the route of administration depends on the mechanistic question being addressed (see Table 2 for routes of administration, with frequency and volume limits). For the models of arthritic pain, understanding of the peripheral mechanisms can be advanced by studying the effects of drugs injected directly into the knee joint (intra-articular (i.a.)), however this route of administration is not relevant to the studies of the models of neuropathic pain. Spinal (intrathecal) administration of drugs permit the targeted application of drugs to specific parts of the spinal cord and brain respectively without exposing the whole animal or the rest of the nervous system to these agents.

Control animals will be generated using regulated procedures. These will either be sham surgical or vehicle control injections in equivalent conditions to experimental animals. Naïve controls are used when previous evidence has shown that these are equivalent to a sham or vehicle control. In these circumstances, the use of shams or vehicle controls cannot be ethically justified.

In a typical experiment, an individual animal will experience habituation and handling, baseline measurement of nociceptive thresholds and/or other behavioural measures (e.g. assessment of anxiety status), followed by induction of an acute or chronic pain state or relevant control procedure. Multiple recordings of nociceptive thresholds will then be collected over a time scale relevant to the model (hours to days for acute models, days to weeks for chronic models). These measurements may take place in the presence or absence of pharmacological interventions administered via an appropriate route, delivered over an appropriate period to the individual experiment (e.g. pre-exposure, or an acute or chronic treatment schedule). Biological samples will be collected for analysis either at multiple time points, or at a suitable predetermined endpoint. Animals will then either be utilised for non-recovery procedures such as electrophysiology, or humanely killed via an approved technique.

What are the expected impacts and/or adverse effects for the animals during your project?

We will use models of inflammatory, arthritic, and neuropathic pain. Adverse effects of these models vary.

The models of inflammation cause some tissue swelling at the site of injection but this doesn't spread to other sites. The inflammation lasts for a few days and causes some changes in movement, but this won't alter the animals' ability to access water or food or to interact with cage mates. The animals have small, but biologically important, changes in their pain responses to a fixed stimulus applied to the site of inflammation.

The models of arthritis and neuropathic pain involve either a short surgical procedure or an injection of a substance (under anesthesia) that causes damage to cells that lead to the injury of the joint or the sensory nerves . The effects of these models last longer (weeks to months) than the models of inflammation, they also lead to sustained changes in pain responses to fixed stimulus applied to the site of injury. These models may also cause some short-lived reductions in weight gain and some reduction in mobility following the model induction, but these resolve after a few days. Models of chemotherapy-induced neuropathy may result in sustained reductions in bodyweight.

The models of pain used are associated with changes in thresholds to painful stimuli which are measurable when stimuli are applied to freely-behaving animals. These are the same tests as used clinically in people with chronic pain. They provide us with very useful data whilst causing minimal distress. If performed at a high frequency, however, the tests may contribute substantially to the cumulative distress of the animal. To minimize this risk, frequency limitations have been laid out for each test (Table 2). The other behavioral tests are also well refined and used in many labs. These tests measure activity or weight distribution on the limbs, they are not expected to cause any harm or stress even when repeated often, no limitations are necessary for the frequency of testing. With measures of anxiety we have selected tests that do not induce anxiety themselves and thus ensure welfare is maintained. Again, limitations to frequency will be adhered to in order to ensure animal welfare is not compromised.

The welfare demands of rodents can be well satisfied in captivity. The welfare of our animals is important for the success of our studies, which would be confounded by undue discomfort and stress of the animals.

The following causes of stress: Use of repeated anaesthesia; repeated nociceptive testing; temporary restraint associated with handling, injections/dosing; administration of pharmacological tools. These effects are short-lived and do not persist long following the procedure.

Potential adverse effects following surgery: wound breakdown, weight loss. Wound breakdown does not happen frequently and doesn't reoccur. Weight loss is generally short-lived.

Implantation of mini-osmotic pump can cause discomfort at the site of implantation, as indicated by excessive scratching or biting at the site.

Administration of substances to induce models of pain can cause a swelling at the site of injury.

Altered gait or limping can occur immediately following model induction up to 7 days following induction of the models of chronic pain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Many of the animals used in this project will have experienced moderate severity, due to the surgical procedures, the manipulations of sensory nerve, spinal cord, or brain function, the behavioural testing procedures, or a combination thereof. Around 1/3 of the animals will be controls for the models and will have a lower severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Pain arises due to complex interactions between cells in many different parts of the body including the peripheral and central nervous systems, the immune system, circulation and endocrine systems. At present these cannot be adequately replicated in vitro. Rats and mice are vertebrates, like humans, and share the basic anatomical and physiological responses to pain that are seen clinically in man.

Pain relies on the integration of noxious information into complex spinal and brain systems which are not present in invertebrates.

Which non-animal alternatives did you consider for use in this project?

The central nervous system in particular is exceptionally complex and something that cannot be replicated in vitro. In silico approaches rely on obtaining large datasets from in vivo studies of chronic pain mechanisms before they are useful. Laboratory rodents are the least sentient species in which these studies can be performed.

Where possible assays of individual neuronal responses will be assessed using primary cell culture based approaches, thereby reducing the welfare burden upon animals.

Why were they not suitable?

Pain relies on the integration of noxious information into complex spinal and brain systems which are not present in invertebrates or in silico models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals is based on our previous usage in the last full year before the pandemic (2019). During this period, the studies we undertook were performed by a similar number of PDRAs and PhD students we will have for the next period and therefore gives an accurate estimate of our capacity in terms of people and laboratory space and the balance between the use of shorter versus longer duration pain models. The experimental techniques to be used are similar to those used previously, providing additional confidence in the number of studies we can undertake per year.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the experimental design assistant, and our previous experience in the design of these types of studies to ensure that the appropriate numbers of animals are used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our previous experience of performing these types of studies in the field of pain research have provided us with valuable information which can be used to perform calculations during the design of research studies to ensure the minimum number of animals are used.

All studies have to be designed and planned well in advance by each experimenter and a written plan with appropriate power calculations presented for inspection by the licence holder and/or deputy. We are therefore confident that with this step included in our procedure we can minimize animal use and optimise the value of the data generated.

Pilot studies are performed where possible during the design stage of major studies. To maximise the utility of each experimental animal, biological tissues not required for our research endpoints will be shared with other researchers where possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Cutaneous Inflammation: Under brief anesthesia to minimize stress, single one site injection into the ventrum between the pads of one hindpaw (s.c.) of either Complete Freund's Adjuvant (CFA (2:1 CFA: Saline)), carrageenan (2%), capsaicin (2%) in an appropriate volume for the age and size of animal as determined from the LASA guidelines as indicated in the protocols. All of these compounds produce tissue swelling but vary in the behavioral sensitization, yet have minimal effects upon the animal's general health and wellbeing. Injection of any substance per se will lead to a short-lived activation of the nociceptors and the pain circuit.

Arthritis: Models of OA are generated under short duration anesthesia by either the injection of monosodium acetate (MIA) through the infrapatellar ligament of the knee (intra-articular injection) or surgery: medial meniscal transection (MNX); destabilization of medial meniscus (DMM). The MIA, MNX and DMM models only involve one limb. In the absence of experimenter-evoked stimulation of the mouse or rat, these models of arthritis do not present any external signs apart from early swelling of the knee and minor locomotor impairment during the first few days following model induction.

Neuropathic Pain : Under brief anesthesia to minimize stress, spinal nerve ligation (SNL – ligation of spinal nerves L5 and L6). SNL is produced by the unilateral ligation of two of the branches of the spinal nerves innervating the lumbar spinal cord (only one limb). SNL induces pronounced, reproducible evoked changes in sensitivity to mechanical stimuli. To minimise surgery pain a local anaesthetic cream will be applied topically. We will also use models that reproduce the changes in pain associated with drug treatment (cancer chemotherapy following the use of paclitaxel or cisplatin).

Cancer chemotherapeutic peripheral neuropathy will be induced by repeated injections of chemotherapeutic drug (paclitaxel or cisplatin).

Animals will undergo threshold testing with mechanical and / or thermal stimuli. Animals will be exposed to a combination of behavioral tests selected on the pain model being studied. Changes in latencies or thresholds are indicative of increases or decreases in sensitivity. Non-evoked behavioral tests may be performed that measure affective states, locomotor activity, and weight bearing distribution.

The duration that an animal will remain subject to a pain model will be depend upon the model that is being studied. Maximum durations are stated for each model and these have been determined based on our previous experience and careful examination of the scientific literature. All animals will not necessarily be maintained for the entire period permitted.

Due to the aim of the research, it is often not possible to use analgesics routinely, as they will alter the processes that we are aiming to model so we can identify new approaches for the treatment of pain in people. In many cases, particularly in neuropathic models, the initial injury and associated responses are key in driving both peripheral and central changes that are responsible for changes in pain sensitivity. The measurements of interest (changes in threshold responses) are surrogate markers of changes in pain processing, modulation of this response before a treatment will increase variability and decrease the effect size of any novel treatment we are testing. However in some cases the injury may cause pain unrelated to the model, e.g. when associated with invasive surgery. Thus, in these cases where appropriate, post-surgical analgesics may be given.

Why can’t you use animals that are less sentient?

Pain arises due to complex interactions between cells in many different parts of the body including the peripheral and central nervous systems, the immune system, circulation and endocrine systems.

These cannot be replicated in vitro. Rats and mice are vertebrates, like humans, and share the basic anatomical and physiological responses to pain that are seen clinically in man. Pain relies on the integration of noxious information into complex spinal and brain systems which are not present in invertebrates.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To ensure clinical relevance of our studies, it is important that these experiments are performed on mammalian species. Rodents have become the ideal choice for pre-clinical research as they have proven to be reliable models of humans in many aspects. As rats and mice have been so often used their anatomy, physiology, and welfare are extremely well understood. This facilitates and expedites research that can be done into the understanding of pain and pharmacology, all whilst being able to ensure that animal welfare is conserved. Our research aims to build upon pre-clinical work performed over the last century, and so the majority of models and tests we plan to use have been extensively

developed, refined, and validated. We aim to use a range of models that reveal unique insights into neurological mechanisms whilst maintaining animal welfare as much as possible. The durations of the pain models vary, in part due to the duration over which individual mechanisms act, and in part due to the nature of the injury. The shortest models are the inflammatory models which usually resolve within a week, whilst the arthritic models are longer lasting, mimicking the clinical problem. The length of the study will be based upon the aim of the study, however some models will be limited in duration to ensure animal welfare is upheld.

We will minimize unwanted pain responses due to potential irritation following repeated dosing of drugs, or post-operative pain due to a surgical procedure. This is achieved through the limited use of repeated injections into the same site and the use of local anesthetic cream (EMLA) at the sites of an incision, e.g. during the generation of models of OA or neuropathic pain. In cases where a recovery surgery is performed prior to the induction of a pain model, systemic analgesics may be given to minimize any pain. In all cases, we will attempt to minimize suffering by ensuring that upon completion of surgery animals are placed in a recovery cage or their home cage with bedding, warmth, and easy access to hydration to ensure that they are comfortable and that their environment is enriched.

Experimenters will closely observe the animals to ensure that they recover appropriately.

We have incorporated best practices for ensuring the welfare of neonatal animals is maintained for a broad range of pain models. In each of the pain models the refinements that have been made for the adults will be incorporated alongside appropriate adjustments for volumes administered. Furthermore, when appropriate we aim to make specific changes that may benefit the younger animals, for example inflammatory substances will be administered to the dorsum of the hindpaw to minimize the impact that the pain model has on the pups ability to develop motor skills and compete for food.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidelines published by NC3R, including the ARRIVE guidelines, as well as guidance published by the Laboratory Animal Science Association (LASA) (https://www.lasa.co.uk/current_publications/).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By attendance of NC3Rs webinars and training courses. We also receive regular updates and latest animal welfare guidance through our Named Competency and Training Officer (NCTO).

128. Pathology and Treatment of Lysosomal and Related Diseases and Brain Tumours

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Therapy, Inflammation, Lysosomal Disease, Brain Tumour, Neurological Disease

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	neonate, embryo, juvenile, adult, pregnant, aged
Sheep	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The purpose of this project is to develop new treatments for lysosomal and related diseases (LSDs), such as Mucopolysaccharidoses (MPS) and brain tumours through better understanding of how they are caused. We want to study how inflammation changes the course of disease and will develop new therapies using this knowledge and test the

quality, effectiveness and safety profile of them in both mice and sheep prior to clinical trials.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Lysosomal and related diseases are inherited genetic disorders which lack specific enzymes that breakdown complex sugars, fats or proteins in a compartment of the cell called the lysosome. These include progressive childhood metabolic lysosomal diseases such as mucopolysaccharidosis (MPS) types I, II, IIIA, IIIB, IIIC, IV, VI and VII, Krabbe disease, Wolman disease and many others. A build up of undegraded by products results in often severe inflammation, blocks to some signalling pathways, damage to organs and in some cases severe damage to the brain resulting in behavioural difficulties and death before 20 years of age. Some of these diseases such as MPSIIIA, and B primarily affect the brain making treatment development very challenging. Brain tumours typically include the most common glioblastoma, which invades the brain and also causes severe inflammation. All of these diseases primarily affect children or young adults and most have very poor existing therapies. As they are individually rare, it is important to find commonalities between the diseases to aid in the development of future therapies. Current treatments are unable to correct the brain damage seen in many forms of lysosomal diseases, especially MPSIIIA and MPSIIIB since enzymes are unable to enter the brain. Bone marrow transplant is curative for a small subset of lysosomal diseases, but not all, and has even been tried for brain tumours with mixed results. Aside from supportive care, there are no effective treatments for these severe diseases and therefore they represent an urgent unmet clinical need especially with regard to treating the brain. Furthermore, each of these diseases provide a huge cost to the NHS, with enzyme treatment for an average child with a lysosomal disease at £250,000 per year. There is a large gap in our knowledge of pathology in these conditions and where disease treatment thresholds lie.

What outputs do you think you will see at the end of this project?

Short-term benefits: Improved information on inflammation in brain tumours and lysosomal diseases, understanding of the role of IL1 in neuroinflammation and behaviour, outcome of phase I/II trial of Stem cell gene therapy in MPSIIIA.

Medium Term: Further development of stem cell gene therapy clinical trials for MPSIIIA and initiation of clinical trials for MPSII and MPSIIIB. Refinement of brain delivery of haematopoietic stem cell gene therapy, improvement of direct brain gene therapy delivery methods in sheep.

Long term: Clinical trial of direct brain gene therapy for MPSIIIC using methodology developed in sheep, development of anti-inflammatory therapies in MPS diseases and expanding their use to other orphan diseases, development of new cell therapies for lysosomal disease, understanding of respective functional roles of inflammatory cells in tumours, development of novel therapies for brain tumours, clinical market authorisation of stem cell gene therapy for MPSIIIA.

We expect to publish our work in scientific journals and at conferences and to be able to generate new patents around methods of treatment -ideally for several diseases at once, and either commercial licences or a spin out company to take these advances forward into patient therapies.

In addition, we will build on our existing tissue bank of samples for collaboration. Our repository of scanned slides provides an avenue for furthering collaborative understanding of inflammatory and disease processes across multiple diseases.

Who or what will benefit from these outputs, and how?

There are currently no adequate treatments for the conditions we work on, as such the main benefits of this project will be the development of novel treatments that could make a significant impact in the lives of these children. The work will improve how we use bone marrow transplant currently and in combination with other treatment strategies which are most appropriate for therapy in the brain and help to bring them to clinical trial more rapidly.

These data will be of paramount importance for the development of 3 novel therapies for lysosomal diseases and brain tumours in the next 5-10 years and in the short-term will inform stem cell biologists, transplant surgeons and immunologists both nationally and globally in the interim with our research findings on stem cell engraftment. The National Institute for Clinical Excellence has begun to reject enzyme replacement therapies, beginning with the Morquio drug Vimizin (later reversed), due to the poor cost benefit relationship of these products (£250K/annum/patient). These costs are not sustainable, thus the gene and drug therapies that we develop are key to solving this crisis.

How will you look to maximise the outputs of this work?

We will maximise outputs through several means. We will collaborate with groups both within the establishment and elsewhere to generate and share knowledge of this diseases and models. We will look to publish all findings, even when unsuccessful. We will disseminate knowledge through conference presentations and publications.

Species and numbers of animals expected to be used

- Mice: 15,000
- Rats: 150
- Sheep: 60

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the least sentient species in which models for lysosomal diseases exist and provide some of the best available models of human disease progression. Mice are typically used between 2 months of age and 12 months of age. Many of the diseases we work with are childhood diseases and therefore working with young mice more accurately represents the human disease. Occasionally we need to use rat models of disease if the mouse models don't adequately replicate disease characteristics. Brain tumour models, especially patient derived tumours implanted into adolescent immunodeficient mice, or genetic models from birth in mice provide some of the most translatable models to human cancers.

Scaling therapies up from the mouse brain to humans is difficult, therefore the use of a large animal model is required to optimise delivery routes and doses for later human trials. Many people are focussed on concentration of gene therapy products and volumes, whilst in confined and easily damaged spaces like the brain, flow rate and achieved pressure may be more important. Sheep provide a good representative model of humans in terms of brain anatomy and structure, blood supply, metabolism and lysosomal storage. Even the density and spacing of neurons in sheep brains better models a human brain than mouse brains. A sheep's brain at 140g, is the same size as a large monkey and is closest in size to children for any available large animal model.

Typically, what will be done to an animal used in your project?

Most animal models are bred from birth with a genetic disease, and many of these are not initially harmful to animals. Over a period of several months models of lysosomal and related disease and immune models start to show behavioural changes, reductions in joint mobility and evidence of inflammation, depending on the disease and most have a shortened lifespan. We usually implant patient derived brain tumours into immuno-deficient mice to make brain tumour models for subsequent treatment and some models are very slow, so we use drugs to make them more rapid to more closely model human disease. Other brain tumour models are initially harmless until the injection into the brain of an activating virus. We closely monitor harmful effects on animals and introduce mitigating measures where appropriate or cull animals where required.

Typical protocols for rodents include:

1) phenotyping to understand disease. Typically 2-3 times for each of the following over the several months lifetime of an animal:, an animal may undergo non-invasive behavioural tests, generally involving brief (10-30 mins) tests with no pain suffering and

distress or food withdrawal for more than 16 hours. Intervals between tests will be at least 10 minutes and the maximum number of individual

tests or trials will be five in any 24 hour period, with no more than 24 tests or trials in a month. Animals may have retinal imaging under anesthesia. During the follow-up period, samples of bodily fluids (blood, saliva, urine) may be taken to analyse the effects of treatments and/or the health of the animal on up to 10 occasions as well as non-invasive imaging interventions on no more than 4 occasions which typically require anaesthesia and sometimes injection of a radiotracer (2-4 hours), contrast agent or light emitting compound. Animals may be culled under general anaesthesia.

2) bone marrow transplant: In addition to phenotyping tests above: A 2-4 month old animal may receive 2 injections to mount an immune response, then receive injections of chemotherapy over 1-5 days to kill off existing stem cells, with a day's break and then receive transplant of gene modified stem cells by injection to replace those killed with chemotherapy. They may then receive further injections of substances to determine if their immune system recognises the new gene as their own. All are extremely brief. Usually all the phenotyping happens after these steps.

3) delivery of multiple therapies: In addition to phenotyping tests above: A 2-4 month old animal may receive up to four therapeutic substances by multiple routes via up to 12 injections, where no more than 2 injections can be given in 24 hours, in combination with bone marrow transplant, in combination with a non-invasive therapy e.g: An animal receives therapeutic drug in drinking water (non invasive), then up to 4 injections of enzyme replacement (each less than 5 mins), then receives injections of chemotherapy over 1-5 days to kill off existing stem cells, with a day's break and then receive transplant of gene modified stem cells by injection to replace those killed with chemotherapy (each less than 5 mins) and then after at least 3 weeks recovery receive 6 injections into the brain of a therapy under anaesthesia (Typically 30 mins to 2 hours). They may then receive further injections of therapeutic substances, such as enzymes. A total of 18 injections in the worst case scenario. Usually all the phenotyping happens after these steps.

4) Immune priming and transplant: In addition to phenotyping tests above: A 2-4 month old animal will receive several injections to mount an immune response, this is expected to worsen disease. All are extremely brief over a few weeks, then may receive injections of chemotherapy over 1-5 days to kill off existing stem cells, with a day's break and then receive transplant of gene modified stem cells by injection to replace those killed with chemotherapy. They may then receive further injections of substances to determine if their immune system recognises the new gene as their own. All are extremely brief. Usually all the phenotyping happens after these steps.

5) Brain tumour implantation and treatment:In addition to phenotyping tests above: An animal will have a tumour implanted in the brain by injection or a virus to activate tumour

growth. An animal may receive up to four therapeutic substances by multiple routes via up to 12 injections, where no more than 2 injections can be given in 24 hours, in combination with bone marrow transplant, in combination with a non-invasive therapy e.g: An animal receives therapeutic drug in drinking water (non invasive), then up to 4 injections of therapeutic drug (each less than 5 mins), then receive injections of chemotherapy over 1-5 days to kill off existing stem cells, with a day's break and then receive transplant of gene modified stem cells by injection to replace those killed with chemotherapy (each less than 5 mins) and then after at least 3 weeks recovery receive usually 1-2 injections into the brain of a therapy under anaesthesia (Typically 30 mins to 2 hours). A total of 18 injections in the worst case scenario. Usually all the phenotyping happens after these steps.

Typical protocols for sheep include:

6) delivery of therapeutic substances: Sheep models may either be bred from birth with a genetic disease, and many of these are not initially harmful to animals or we use normal sheep. A 2-4 month old animal may receive therapeutic substances by multiple routes, but mainlyvia up to 10 injections into the brain, e.g: An animal receives 6 injections into the brain of a therapy under anaesthesia, with one supplementary intra spinal and two cerebrospinal fluid delivery (brain spaces) injections (Typically 3-7 hours). During the follow-up period, samples of bodily fluids (blood, saliva, CSF) may be taken every 2 months or so to analyse the effects of treatments and/or the health of the animal as well as non- invasive imaging interventions on no more than 4 occasions which typically require anaesthesia and sometimes injection of a radiotracer (2-4 hours), contrast agent or light emitting compound. Typically these are spread over the several months or years lifetime of an animal. Animals may be culled under general anaesthesia.

Animals will typically be injected with a range of drugs/treatments and followed up for up to a year. Animals are always monitored closely throughout the length of the experiment to ensure good health and any signs of pain, distress or ill-health are addressed accordingly (pain-relief, antibiotics, creams, etc). At the end of the experiment the animals are humanely killed and organs are typically harvested for further analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Rodents

Expected adverse effects include weight loss (weight loss will not exceed a humane endpoint of not more than 20% of starting bodyweight), pain following surgery, lethargy and abnormal behaviour.

Animals can experience stress due to restraint and handling which will typically resolve by the end of the procedure. Sometimes food withdrawal for up to 16 hours prior to a test may be necessary for one or two behavioural tests which can cause stress, again limited to the duration of the test. Typically behavioural panels will be performed up to 3 times separated

by several weeks. Where animals require anaesthesia, they will experience transient discomfort from needle insertion and/or anaesthetic injection or inhalation of gaseous anaesthetics. Animals may undergo anaesthesia on up to 4 occasions for imaging , up to 10 occasions for retinal imaging and once for intracranial therapy or tumour implantation. Sometimes, during surgery the animal's breathing can seem laborious, this typically resolves by the end of surgery. Sometimes seizures or brain bleeds can happen in the initial hour after surgery. There is also potential for infections. Delivery of chemotherapeutics to mice (usually 5 days of drug) prior to bone marrow transplant will make mice quite sick, as is the case in patients with a depressed immune system and low blood counts for several days leading to lethargy and weight loss (weight loss will not exceed a humane endpoint of not more than 20%) during this period before the transplant takes.

Sheep

Some possible pain during/after surgery, indicated by teeth grinding. Occasional mild adverse effects of surgery, such as transient tremor or partial facial paralysis. Typically all of these resolve within 48 hours. Sometimes seizures or brain bleeds can happen in the initial hour after surgery. Anaesthesia on no more than 5 occasions; once for intracranial surgery once and four occasions for imaging. There is also potential for infection. Sheep are typically housed in pairs to avoid distress.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For mice, expected severities will be mild to moderate, In breeding protocols 50% of mice will experience subthreshold harms, 40% mild and 10% moderate. In experimental protocols 10% will be mild and 90% moderate. Overall, as most mice fall into breeding protocols - we expect 40% of mice to be subthreshold, 30% of mice to be mild, and 30% moderate. Ffor rats it will be mild to moderate – we expect 70% of rates to be mild and 30% moderate and for sheep it will be mild to moderate. We expect 10% of sheep to be mild and 90% of sheep to be moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The conditions we work on are complex and are influenced by a host of different factors. Many of these conditions are neurological in nature but also have non-neurological components often including heart problems, bone disease and eye degenerations to name a few. As such, non-animal alternatives, such as cell-based tests, may not be appropriate as they can only provide limited results. The mouse models of these conditions mirror the human disease closely, giving us physiological data, such as cell- cell interactions and immune system dysfunction, which cannot be achieved using cells in a dish, nor with zebrafish models. We need to assess that the therapies we develop have the ability to cure all of the affected organs, especially the brain, so computer based assays and cell culture cannot predict outcomes. There also are no appropriate methods to avoid the use of animals to assess cell engraftment after bone marrow transplant of the delivery of gene therapy vectors to the brain. Our therapies are assessed with a variety of outcome measures such as behaviour and tissue sampling which can only be achieved with animal models. When testing our therapies for direct treatment of the brain, it's necessary to use an animal model whose brain is comparable to the human brain in size.

This cannot be achieved by any other method therefore it necessitates the use of a large animal. Sheep are one of the least sentient of these.

Which non-animal alternatives did you consider for use in this project?

We use cell-based tests in the lab to replace animals where possible (e.g. Cell culture assays, High Content Screening technology), which we use to reduce drug or gene therapy candidates to viable numbers for in vivo testing by aiming them at specific characteristics -such as blood brain barrier penetration and cell uptake. We have used agarose phantoms to model brain distribution, but this is not very predictive of in vivo outcomes. There are no suitable in vitro assays or alternatives that are therefore relevant to distribution, delivery of products, immune system responses, brain and behavioural outcomes.

Why were they not suitable?

No adequate tissue culture models of the complexity required to examine tissue and brain repopulation after transplant in living organisms exist. In order to better predict what treatments or stem cells will prove to be effective and safe – we require animal models to test these approaches. The brain is incredibly complex, and we often use behavioural evaluation of mice with disease to assess the effect of therapy which is impossible in tissue culture and/or computationally.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have based these numbers on our previous experience working with these models and the numbers used on previous licences. Around 40% of our rodents will be used in breeding only and most of our rodent lines breed as heterozygotes yielding the proportion of one unaffected, two mixed and one affected mouse - and the remainder will go into protocols. Typically we will maintain up to 5 rodent lines at any time, the remainder being kept as frozen stock. For example, we have over a decade worth of experience performing bone marrow transplant experiments in mouse models of orphan diseases. From this, we can estimate that we will typically need 8-10 mice in each group to see differences between treated and untreated mice (by analysing the mouse's behaviour and markers in bodily fluids/tissue) and we would typically have 3-5 groups. We also have an accurate idea of how many experiments we would like to complete over the course of the 5 years that this licence will run.

We expect to evaluate 10-12 new substances of which 2-3 will be taken forward to clinical trial.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use statistical methods to calculate the number of animals needed for our experiments. We will also perform as much work as possible in non-animal models, such as cell culture experiments, to further reduce the number of animals needed.

We often use repeat imaging to reduce the number of animals that we have to cull at set time points This means that we reduce wastage and allow paired data analysis to reduce between subject variation. We also usually share controls between experiments where possible by running multiple experiments in parallel.

We often use repeat imaging to reduce the number of animals that we have to cull at set time points and usually share controls between experiments where possible by running experiments in parallel. We have extensive experience of working with animals and performing these kinds of studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will breed animals in such a way that the number of animals from each mating is optimised and we will collect data throughout the lifespan of the animal to generate the maximum amount of data and reduce the number of repeat experiments needed. Were possible we will utilise imaging and sampling techniques throughout the lifespan of the

animal, reducing the need to sacrifice animals. We also have a number of collaborators, therefore, maximum use is made of animal tissues across a number of different projects.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

To achieve the objectives set out in this project licence we will need to breed a number of different mouse models. These will include transgenic mice expressing marker proteins (allowing tracking of various cells following transplantation) as well as the mouse models of mucopolysaccharidosis (MPS) type I, II, IIIA, IIIB IIIC, MPSVI, Wolman disease, Fabry, Krabbe and Gaucher. These are the best mouse models of these progressive childhood metabolic diseases available, are the least severe models, and are used in preference to naturally occurring cat and dog models of the diseases. Each generates a distinct disease and despite similar substrates stored display different behavioural and biochemical outcomes. Comparisons of these models will help us to understand which components are important in each diseases' progression. These models will be used to examine how the bone-marrow- derived cells travel to the brain and also to test the therapeutic effects of the cells, particularly gene therapy modified cells to express the missing enzyme.

Immunodeficient mouse models will be used to study how human stem cells can repopulate organs such as the brain. Each model knocks-out different immune cell types – allowing us to see which are important in aspects like tumour progression. These mice will also be used as they have the ability to grow human brain tumours when they are injected into the head. Disease specific models crossed onto an immunodeficient background, allow the use of human cells to treat the model.

We need to use a large animal model to scale therapies from mice to humans. This is because the mouse brain does not replicate the structure, blood flow or density of the human brain and therapies in mice sometimes dont predict accurate dosing or delivery routes for therapies. A sheep's brain is as large as a large monkey and has a similar structure, blood flow metabolism and density to humans. It also shows lysosomal storage therefore it is the most refined model for this purpose, while being less sentient than monkeys.

Why can't you use animals that are less sentient?

Rodents are the least sentient species in which models for these orphan diseases exist and provide some of the best available models of human disease progression as we have characterised previously. Other models, such as zebrafish, are inappropriate as abnormal behaviour is a characteristic of the diseases we research and zebrafish poorly model these aspects. Although we could start by treating zebrafish to show proof of mechanism, this would not reduce the number of mice required to demonstrate behavioural changes when we deliver a therapy and we typically show that enzyme works in cell culture in advance of any mouse studies. Also it can be difficult to test therapies in zebrafish and, as many regulators do not allow studies in zebrafish prior to clinical trial, we would need to use mouse models anyway so the use of zebrafish seems an inappropriate use of animals. The brain of a sheep is structurally, in terms of blood flow and disease modelling closest to that of a human child compared to other large animal models and is the least sentient of these with large brain size and, therefore, will be used for scaling therapies from mice to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We always look to improve the way we perform procedures. For example, we use chemotherapy to prepare mice for bone marrow transplant in place of irradiation, with much improved mortality rates (now less than 1%) and reduced adverse effects on health to mice overall. As a part of this project we are looking at other chemotherapeutics that may improve this further.

Our mouse intracranial surgery is extremely well tolerated (less than 1% mortality) and this is in part due to the efficiency with which we can complete the procedure given our experience with the model of the procedure (typically under 20mins).

We are able to use image guided sheep intracranial surgery using a CT scan of the sheep brain to create a 3D image of blood vessels prior to surgery and mapping to the skull of the sheep, which is another innovation from patients allowing safe planning, and significantly reduces the time and risks of intracranial surgery in sheep. We have a less than 5% mortality rate in sheep with this technique, which would be much higher without it.

All procedures are undertaken using good aseptic technique to minimise the risk of infection. Post- operative care will involve constant monitoring in the weeks and months following procedures. Animals will be weighed and assessed for pain and distress. Analgesia (pain relief) and other treatments will be used if necessary and in consultation with the vet.

We always keep up to date on the latest guidance and will undertake training when required to improve how we handle and perform procedures on animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All our experiments will be conducted following PREPARE guidelines, ARRIVE guidelines and OECD protocols.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We liaise with colleagues in the animal facility within the establishment who keep us up-to-date on advances in the 3R’s via a monthly newsletter. We also access the NC3Rs and other welfare driving bodies websites for training information. We will also seek to attend seminars and other educational events to further this knowledge as well as reading publications and outputs of colleagues

129. Role of Phosphorylation and Ubiquitin Components in Mitochondrial Damage in Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

mitochondria, Parkinson's disease, genes, signalling pathways, neurodegeneration

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To uncover the cellular pathways that sense and respond to mitochondrial damage with a specific focus on gene components that are mutated in Parkinson's disease and related brain and metabolic diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Parkinson’s disease and related neurodegenerative disorders are incurable disorders of the brain causing significant disability and death worldwide. There is an urgent need to

advance knowledge on the basic mechanisms of these disorders in order to develop disease modifying therapies which are not currently available in the clinic. Over the last 10-15 years we are beginning to understand the genetic basis of rarer inherited forms of Parkinson's and approximately 20 genes have been identified in which mutations cause the loss of defined sets of nerve cells leading to the debilitating symptoms experienced by patients. Until recently the cellular events that occur as a consequence of Parkinson's-linked gene mutations was not known, but we have been studying two genes, called PINK1 and Parkin, and our research has found that PINK1 and Parkin operate together to control the elimination of damaged mitochondria, known as mitophagy. The exact details of this process remain to be worked out under physiological conditions which is a crucial step in providing impetus and rationale for developing drugs that exploit this understanding to treat Parkinson's. Our work has shown that mutations in PINK1 and Parkin lead to a buildup of damaged mitochondria that is detrimental to the health of cells triggering brain cell loss and Parkinson's symptoms. There is accumulating evidence that mitochondrial damage also occurs as a result of mutations affecting other Parkinson's genes including LRRK2 and GBA1 but how this occurs is not known is a major focus of our future work. My research involves making defined changes in the equivalent genes in mice, investigating what the molecular consequences are and determining how they impact on mitochondrial function, cell physiology and neuronal integrity. This information could identify new biomarkers (biological molecules found in blood; other body fluids; or tissues that indicate an abnormal process or disease) and therapeutic strategies for Parkinson's disease and related disorders.

What outputs do you think you will see at the end of this project?

This project will uncover fundamental new knowledge on the molecular, biochemical and physiological function of the key genetic pathways involved in Parkinson’s disease with a major focus on pathways controlling organelle health. This increased understanding will contribute new knowledge to better diagnose and treat Parkinson’s disease.

Who or what will benefit from these outputs, and how?

Currently there is no cure for Parkinson's disease or any objective test for diagnosis and monitoring progression. Research findings from the project may stimulate new ideas in diagnostic biomarkers and therapies which would be transformative for patients with Parkinson's disease. In that regard we anticipate that our findings would be of interest to biotechnology and pharmaceutical companies to stimulate new translational projects that build on our findings to counteract the impact of Parkinson's gene mutations on mitochondrial damage.

How will you look to maximise the outputs of this work?

The research findings from the project will be published in fully open access journals freely accessible to the whole scientific community.

The work will also be presented at scientific conferences and at patient stakeholder meetings as part of Patient Public Involvement (PPI) related activities that are embedded in our Parkinson's research framework within our institution.

The work will be discussed with the industry partners currently collaborating with our institution.

Species and numbers of animals expected to be used

- Mice: 19000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The ease with which specific genetic alterations can be introduced into mice, and the similarities between their nervous systems and our own make them the ideal species in which to investigate the basic mechanisms of mitochondrial damage and neurological function.

The complex structure and architecture of neurons and their organellar, metabolic, mitochondrial and signalling networks cannot faithfully be replicated using current in vitro systems. Therefore the knowledge gained from mouse models in vivo will have more physiological relevance for our understanding of the human disease mechanisms.

Parkinson's disease is a degenerative disorder of the brain in which a fully formed nervous system undergoes progressive neurodegeneration. We would therefore choose to study mice in the adult stage to understand the mechanisms governing brain physiology and behaviour relevant to Parkinson's disease.

The mice system also enables the study of physiological brain cells that can be cultured on a dish. These would be generated from embryos in which embryonic brain tissue is at a pluripotent stage and has not fully differentiated (which means that the cells still have the ability to change into different cell types) and whereupon neural precursor cells (cells that will become nerve cells when they finish changing) can be isolated and induced to develop into distinct neuronal cell types that would be relevant to understand Parkinson's disease.

Typically, what will be done to an animal used in your project?

The majority of animals used in the project will be bred and allowed to age with no other interventions. These animals will be very closely monitored for signs of reduced weight gain or other signs of adverse welfare and will be humanely killed if they become unwell.

What are the expected impacts and/or adverse effects for the animals during your project?

In general, most genetic models used are not expected to show significant signs. However, in using Parkinson's models for ageing, signs may occur and animals will be monitored closely for early signs of movement impairment such as changes in how they walk or are able to perform other activities such as neglect of grooming. Such animals would be humanely killed immediately to minimise suffering and brain and other tissues isolated for biochemical and molecular analysis. A subset of animals may be given non-toxic compounds to alter the activity of Parkinson's gene function and these would be given in food or water, by gastric tube or injection. These compounds would not be expected to cause significant adverse effects and their administration will be performed by trained and competent personnel to reduce discomfort. We will also perform tests that tell us if the animals are less able to perform natural voluntary movement or are less coordinate than usual. These tests of behaviour are not expected to cause harm.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The vast majority of animals will be housed for less than one year (our aim is ~6 months), either for breeding or to allow ageing to a particular time-point where they will be humanely killed to allow tissue analysis post-mortem. Animals are not expected to show any clinical signs of illness in this time and so almost all would be classed as sub-threshold for severity (less harm than a single injection). Some animals may show some slight changes in how they behave or move, and these will be of mild severity. The protocols for this therefore are classified as "mild" to cover the animals who show some signs.

A small number of animals will be aged and so kept for a longer time for experimental analysis of post- mortem tissues from older animals or to allow behavioural analysis for neurological changes (that may be age-dependent and so need to be done in animals of difference ages, including older ones).

Although only slight changes in behaviour or movement are expected in older animals, there is the possibility that some animals may show more noticeable clinical signs and so the protocol to cover this work will be classed as moderate severity, although most animals will again only be likely to reach a mild severity.

A small number of animals will undergo administration of compounds and/or sampling for experimental analysis. This will be moderate severity in case of unexpected adverse responses to the compounds.

Based on our previous work >95% of all animals used will have no higher than a mild actual severity and of these the vast majority will actually have no suffering over the threshold for regulation (and so will be reported as sub-threshold).

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There is still no reliable and physiological method to grow and replicate functioning organs such as the mammalian brain outside the body in a dish. Organs such as the brain exhibit complex architecture of different cell types that cannot grow in isolation. Furthermore, brain disorders including Parkinson's disease exert their cellular effects on different types of neuronal cells and brain regions. Therefore, mouse models are required to study the function and biology of Parkinson’s genes in the context of their role in brain and mitochondrial homeostasis and understand how mitochondria are influenced by the organ environment within an intact animal. The effect of compounds including kinase inhibitors on mitochondrial biology and motor function are also more appropriate to be performed in mouse models. We will also culture primary neurons and other brain cell types from mice that are mature and enable study of fully adult differentiated cell types. The knowledge gained from both the in vitro and in vivo work would be expected to lead to follow up studies that could be undertaken in an in vitro system rather than in animal models. My laboratory has expertise in studying protein kinase signalling in human cancer cell line models and using in vitro methods such as bacterial expression of proteins and enzymology assays. We also collaborate with clinical researchers around the world who have access to human genetic studies and databases wherein we can collaborate with them to determine whether components discovered in our systems are relevant to human disease gene pathway findings in human patients.

Which non-animal alternatives did you consider for use in this project?

Genome editing using CRISPR/CAS9 work in human cell lines and/or E.coli expressed recombinant protein-based studies.

Why were they not suitable?

As described above, the complex structure and architecture of neurons and their organellar, metabolic, mitochondrial and signalling networks in tissues such as the brain cannot faithfully be replicated using these in vitro systems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals that I have estimated is based on the numbers of mice we will need to breed and generate in the next 5 years for our work. This has been achieved through ascertainment of the numbers of mice we have used in the last 5 year for select projects and statistical analysis combined with experimental design to use the fewest number of mice to obtain scientifically rigorous results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I consulted with previously published results from the literature and taken into consideration our previous experience to select sample size and numbers of mice needed to make sure we are using the optimal conditions for any treatments we use. The design of experiments in this project mirrors previous experience where we seek advice on the experimental design and analysis from local scientists specialising in study design and data analysis to ensure the minimal number of animals are used to gain statistically meaningful results. Where applicable we also make use of the the open access Experimental Design Assistant online tool from the NC3Rs website.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Optimisation of mouse numbers will also be based on previous experience and reported study plans recorded in our in-house system. We will consult extensively with the literature for previous published studies to ensure we are following the optimal conditions and treatments used. Where published studies are not available, we will perform preliminary studies with small groups of animals to identify the best conditions and this will allow us to utilise the least numbers of animals. We have developed novel and sensitive monoclonal antibodies to probe the PINK1 and Parkin signalling pathways that has helped reduce the numbers of mice needed to ascertain their role in mitochondrial damage pathways.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mouse models during this project since it is a mammal and the majority of cellular functions are highly conserved between mice and humans. Previously published research in mouse models has also demonstrated their utility to study complex mitochondrial regulation in tissues and signalling mechanisms and motor function that recapitulate the Parkinson's disease process. Such insights have not been possible to achieve using in vitro cell studies or human and other non-human surrogate models. The robustness of findings from mouse models will also be critical in guiding the research field and pharmaceutical companies to develop new therapeutic strategies for the human disease.

Many mouse lines of Parkinson's disease to be used in this project are already available and so new research will be undertaken building on these existing models, thereby minimising the need to generate new models. For any new lines needed in the project, we will consult with national and international animal resource centres to use lines already generated.

Why can't you use animals that are less sentient?

The unique nature of Parkinson's disease in humans requires dissection of the mechanisms in mammalian systems. Many key aspects of mice brain physiology relevant to Parkinson's disease such as Dopaminergic neuron architecture and networks has been shown to be well conserved with the human brain. These are not well conserved in less sentient animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our project will not employ surgery and have been designed to cause minimal suffering and distress to animals. Animals will also be closely monitored throughout their lives for signs of distress to ensure their welfare is not adversely affected.

We will also appraise and monitor our planned methods and techniques for ways to improve these from an animal welfare standpoint. We will also keep close ties with other centres and the literature to keep abreast of any new reported adverse effects linked to our mouse lines and will consult with appropriate veterinary counsel on ways to mitigate these.

We may need to generate or import new GA lines for which the precise adverse effects of the genetic alterations are not known. Some lines may be embryonic lethal or lethal before adulthood and such lines will be made as conditional knockouts or be maintained as heterozygotes (only one out of two copies of a gene affected which means that animals are less affected by the genetic change), after approval by the local Welfare and Ethics Committee. Animal showing overt clinical signs that would cause an increased level of severity to that expected will be culled.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow published best practice according to the NC3Rs ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I have registered and an account at NC3Rs and will receive regular email updates including the NC3Rs newsletter. I am an active member of the mouse user group at my institution which provides further ways for me to remain informed on advances in the 3Rs.

130. Hypoxic Signaling and DNA Repair in Zebrafish

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

DNA repair, HIF signaling, Cancer, Neurodegeneration, Aging

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The repair of our DNA is of high importance for our long term health, improper repair is at the basis of a variety of diseases, including cancer, neurodegenerative diseases, and aging. DNA repair has been studied in vertebrate cells, however the results of such experiments require further verification in animal models, as cells in culture cannot adequately represent the complexity of an entire organism.

An important second objective is to study how one particular signal interfaces with DNA repair, this signal is activated when oxygen levels are low, and is mainly transmitted by Hypoxia Inducible Factor (HIF).

We published the first model in zebrafish which mimics HIF activation. We have shown that activation of HIF surprisingly protected the larvae from DNA damage and thus may

have benefit in prevention of tumours or side effects from cancer treatment. In addition, we have evidence it may alleviate the effect of genetic diseases that are caused by defective DNA repair.

Continuing with our zebrafish mutant, we will study how HIF may lead to this protection. Our studies will include:

- Analyse the consequences of loss of various DNA repair genes or activation tumour promoting genes, and the effect of HIF on these.

- Establish new methods/models to assess DNA damage and their consequences, in larvae or adults

- Test possible therapeutics on our models with a focus on HIF.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer, neurodegeneration and ageing are major problems in our society, and are to a significant part driven by DNA damage. Understanding how this occurs and how these could be prevented could have important positive implications for patients. It is clear that although studies on cells in culture have contributed enormously to our understanding of DNA repair, effects on organismal level are hard to predict and require dedicated models.

What outputs do you think you will see at the end of this project?

A detailed understanding of how HIF can protect our DNA against damage, and how various forms of DNA damage affect the development of zebrafish.

Establishment of the zebrafish as a lower vertebrate DNA repair model. In particular, the creation of various genetic or transgenic models leading to cancer, neurodegeneration or aging that can be used to test interventions. New ways to create DNA damage in zebrafish and readouts to assess the effectiveness of interventions.

Our work will be published in scientific journals and presented at suitable conferences

Who or what will benefit from these outputs, and how?

Benefit will be to scientists. For instance, scientists studying DNA repair in cell culture often require confirmation of results in living animals. The development/use of zebrafish will be more cost effective, faster and in line with the 3Rs principles.

Longer term our work may help to identify drugs that may be used to treat patients. For instance, our current work suggests that one drug acting on the HIF pathway could benefit patients with a rare cancer syndrome.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this work through both conference presentations, collaborations with other laboratories focusing on HIF and DNA repair and publications of our findings.

We have ongoing collaborative studies with academic colleagues in the UK and abroad. We have shared transgenic reagents and protocols that were created as a result of current studies and co- authored a number of research publications that were enabled by the use of our materials. Where needed, we will identify or seek out new scientific collaborators, for instance to take drug studies forward.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 19500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In line with the 3Rs we aim to perform the great majority of our studies in zebrafish larvae that are neurophysiologically less complex that adults and are not protected by law (before 5.2 days of age).

For addressing the phenotypic consequences of some genetic alterations we will need to raise some fish to adulthood, to observe relevant phenotypes. Again, the use of zebrafish means that we use a vertebrate model with a low neurophysiological complexity as compared to other commonly used animals: mice or rats. We need adults because aging, tumourigenesis and neurodegeneration are often not fully "developed" before the start of legal protection of zebrafish embryos (5.2 days of age).

Typically, what will be done to an animal used in your project?

Typically, we will take an embryo that has a particular genetic alteration (e.g. a mutation in a DNA repair gene) and a normal control embryo, and expose them for instance to a drug and combine this with a treatment to damage DNA. Before the larvae reach the age of legal protection, we will analyse its development using a variety of readouts.

In some cases, we will create a novel genetic alteration and want to study the impact of this in development of the larvae. If no strong early defects are seen, we will raise some fish to adulthood and assess whether these animals develop abnormalities later in life, for example behavioural defects, signs of cancer, or premature aging. Animals may receive an additional DNA damaging treatment (e.g. radiation) to bring out latent defects, if needed.

What are the expected impacts and/or adverse effects for the animals during your project?

When considering the legally protected animals that are more than 5.2 days old, we may expect tumour formation, behavioural abnormalities (as a result of neurodegeneration) or premature aging. When experiments on adults are required we will carefully monitor such signs, we will not allow these to continue for more than necessary.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Almost all of the experimental animals in an experiment will experience mild discomfort, a small amount (10% of animals used) of fish may experience transient moderate discomfort (e.g. as result of a radiation treatment).

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of our project is to verify findings that were done in cell culture in animals. This is essential, as cell culture cannot faithfully represent the in vivo environment where different tissues and cells interact with each other (e.g. nerve cells, immune cells and blood flow). In addition, cell culture selects for rapidly dividing cells, and occurs under high oxygen levels. The cells that "fill the dish first" will be selected for. We have found that this may mask the requirement for HIF to prevent DNA damage, and have found that although certain DNA repair genes were proposed to be essential, fish with defects in these genes were viable and without obvious phenotypes.

Which non-animal alternatives did you consider for use in this project?

We are using cell culture to inform our work, this is the basis for our in vivo experiments.

Why were they not suitable?

Animal work is important and essential because in the past we have found clear discrepancies with cell culture results. (eg Zaksauskaite et al., 2021, Kim et al., 2021). Cell culture cannot faithfully represent the in vivo environment where different tissues and cells interact with each other (e.g. nerve cells, immune cells and blood flow). In addition, cell culture selects for rapidly dividing cells, and occurs under high oxygen levels. The cells that "fill the dish first" will be selected for. We have found that this may mask the requirement for HIF to prevent DNA damage

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The bulk of our adult fish is required to safely maintain the genetic integrity of our lines (8000) and generate new genetically altered lines (~3000) over 5 years (the majority of other protected animals will be post-d5 larvae that will be grown to max. 10dpf). As sex ratios are variable and often a small number of the fish carry the correct genotype. Our design accounts for issues such as number of fish required to safely maintain breeding stocks, breeding new generations of fish at an appropriate age to prevent welfare issues that can arise in ageing populations, and generation of sufficient alleles of new mutants for robust genotype/phenotype correlations. In addition, where appropriate we will use the ZEG or similar genotyping protocol (see References section), which will allow us to raise to adulthood only the genotypes required (e.g. only heterozygous carriers from an outcross), which can reduce the number of fish used in certain experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Where applicable we will use the NC3R’s Experimental Design Assistant, to consult on sample sizes for individual experiments to ensure the correct number of animals required is used. For some experiments we have sufficient pilot data on variation to perform priori power calculations to calculate group sizes. We will not initially exceed the predicted group sizes required to detect a 40% difference

with 80% power (alpha of 0.05 as per convention). We will increase group sizes only if greater power is required, if the variance is greater than in pilot studies, or where the biological effect to be detected is less than 40%. Where we obtain data over time from the same animals, we will use statistics appropriate to repeated measures. The use of the same animals over time greatly reduces the animal number required (compared with groups of animals sacrificed at each timepoint as occurs in many mammalian studies).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will strive to use pre-d5 larvae rather than older (protected) larvae/fish where possible. We will also incorporate ZEG genotyping or consider further non-harmful embryonic genotyping methods to reduce the number of fish to be raised. In addition, we will consider using CRISPANT approaches to create loss-of-function mutants without having to establish mutant lines. This could reduce the number of fish needed to maintain breeding stocks.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our work mainly uses larval stages to study DNA repair and HIF signaling. Numerous experimental methods have been published or have been developed by us to create DNA damage, or to provide readouts. These larvae are not protected by law and suffering will be kept to a minimum.

Some of our experiments may require the use of older zebrafish to take advantage of their less complex neurophysiology compared with higher vertebrates (e.g. mammals).

Why can’t you use animals that are less sentient?

We have considered other models e.g. Drosophila or C.elegans however, these simple invertebrate organisms are not suitable to study the important effects HIF signaling has on the circulation and immune system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are always looking for methods to reduce welfare costs. However, our current protocols already are expected to have at most a mild impact on our fish. We will strive to find markers that are predictive in larvae for adult phenotypes: For instance, aging may be linked to cell senescence, we plan to use a new senescence marker p21:GFP which might be predictive in larvae and reduce the need for raising animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will work according to the suggestions provided by Aleström et al. 2019 with respect to fish care.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am actively involved in our University in AWERB and get some information via this channel. I plan to attend 3R training events, as I have done in the past. Our aquarium team organises regular meetings where new developments are communicated. I have actively promoted adoption of the ZEG genotyping protocol. We published a methods paper on embryonic fin clipping as a way to reduce raising of unwanted genotypes. Currently, we are working on using CRISPANTs to avoid the need for keeping mutant animals.

131. Development of an Advanced Home Cage Phenotyping Technology for the Mouse

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Telemetry, Home Cage Monitoring system, Phenotyping, Epilepsy, Sleep disorders

Animal types	Life stages
Mice	juvenile, neonate, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop an advanced mouse characterization platform which will combine the wireless telemetry systems (to measure central and peripheral electrical activity) with the Home Cage Monitoring (HCM) (to record behaviour and activity).

The integration of these systems will improve the quantity and the quality of data obtained from mouse genetic research, which can inform on gene function within intact physiological systems in the mouse.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Genetic disease mechanisms are intensively investigated in the many thousands of genetically altered (GA) mouse strains produced every year. In recent years, techniques to generate GA mice have become more sophisticated, easier and faster, delivering multiple alterations in the mouse with increasing similarity to those changes seen in human patients.

This increased ability to model genetic diseases drives the need to improve the ways in which we investigate gene function by generating efficient and reproducible tests. The improvement of these techniques is essential for the study of mouse strains, in which observable characteristics (called phenotypes), which relate to human disease symptoms, are measured.

We offer support to individual research laboratories to study and characterize specific genetic modifications in their models of interest. We are therefore striving to develop the most robust, effective and advanced methods for the study of mouse phenotypes in order to improve preclinical research.

In recent years, several advances in technology have led to refinements in phenotyping, including more computerized testing which decreases the interactions with animals, increases objectivity and improves the welfare of the animals. The aim of this work is to establish a more advanced phenotyping system that integrates wireless electrophysiological recording with HCM in mice, allowing concurrent recording of activity and other behaviours with electrophysiological parameters.

We need to be aware of the many technological challenges that can be faced when developing this new phenotyping method. For instance, interactions between the two devices may lead to a decreased signal, or there may be incompatibility between the two systems or behavioural changes in the mice implanted with the electrophysiological system that may impact on the HCM measurements. In addition, even if the systems are compatible, there may be challenges involved in combining the two signals, as they are simultaneously recorded with different software and over long time periods.

Wireless telemetry systems leaves the mice free to move around the cage with no limitations or restrictions to their movements. This is the most refined method of measuring electrophysiological signals and it is an important improvement on the use of devices that restrain the mice (tethered systems). Telemetry can provide details of brain cell-cell interactions or muscle activity and therefore can be used in a huge variety of disease models. The telemetry system can provide information to investigate the pathways involved in neurological disorders in both the central and peripheral nervous systems such as sleep disorders, epilepsy, Amyotrophic Lateral Sclerosis, Alzheimer disease and neuromuscular disorders.

The HCM system allows passive phenotyping in the mice's own cage without the need to move animals to unfamiliar environments, with no interaction with the experimenter and with no alteration of the social group in which the mice are housed. HCM systems include the tracking of individual animals using radio-frequency identification with video monitoring. The combination of the telemetry system with the HCM system would give the advantage of integrating electrophysiological recording, behaviour and activity continuously and for a long period of time in the same mice, decreasing the number of animals used for the study.

Our aim is to apply the new advanced phenotyping system to GA mouse strains with either epilepsy or sleep disorders, in order to integrate visual appearance, behaviour and functional data. The goal is to establish this technology in wild-type mice and to obtain initial data for the further study of disease phenotypes.

Disturbed circadian rhythm and sleep patterns are associated with several brain diseases that affect behaviour, such as depression and bipolar disorder. The consequences of this disturbance include neurological problems, reduced immunity and metabolic disorders. Epilepsy and increased seizure numbers are also associated with several neurological dysfunctions, such as Down's syndrome and Alzheimer's disease, with no known reason for the appearance of these symptoms.

Within this study, we also aim to develop new refined and advanced phenotyping technology that can be applied to an extensive range of disease studies. By concurrently recording electrophysiological data for seizures and/or sleep, we intend to use the acquired video analysis to: a) identify behaviours/changes in activity that precede, are present during, or follow these physiological states, and b) use this information (including the shape and form of the animal) to train neural networks or other machine-learning methods to automatically recognize periods of time in which these states are occurring. This has the eventual aim of being able to detect these states in HCM without the necessity to implant telemetry devices.

This will help us to deliver better preclinical research, with the aim of improving the phenotypic study of disease pathways and of translating results to potential therapies for diseases in humans.

What outputs do you think you will see at the end of this project?

This project will improve and develop our phenotyping techniques, providing the ability to study mouse models in the most comprehensive way and without interaction between the experimenter and the mice.

We will deliver a new combination of technologies, with a protocol for recording baseline data on wild- type and GA lines and a protocol for data analysis. We aim to improve the quality, quantity and reliability of data output, thereby greatly improving the characterization of GA mouse strains.

Our goal is to integrate the telemetry data with the data obtained from the Home Cage Monitor (HCM) system that records mouse movements and behaviours. The aim is to record all the data at the same time to investigate any signal interference between the systems. We will develop a more refined protocol to increase the complexity of our phenotyping data.

The main benefit will be to obtain as much data as possible from individual mice in longitudinal studies thereby reducing the number of animals used and enabling the monitoring of all phenotypic changes in the same animal over time. These protocols will be applicable to mouse models of human diseases. It is expected that the detection of electrophysiological signals of physiological disturbances such as seizures, in combination with the analysis of patterns of activity and longitudinal behavioural assessment from the HCM system, will provide opportunities for the development of earlier humane end points, by monitoring animals much more comprehensively and for 24 hours a day.

The combined data will also produce video recording, in which behaviours preceding, during and after physiological states and seizures can be recorded. It is intended that, in collaboration with data scientists, we will develop automated systems that will be able to detect alterations in the functions of the organism without the need for telemetric implants.

This refined assessment will be available to the wider scientific community through publications. The combined dataset will be available for machine-learning applications and in the future will form the basis of a service licence to provide these phenotypic analyses to UK biomedical researchers.

Who or what will benefit from these outputs, and how?

By applying a more extended analysis in wild-type mice, we will establish the baseline data and the protocol to translate the analysis to GA mice, with the advantage of studying gene functions in a more comprehensive way.

We will set up the new phenotyping technology in GA mouse strains that model sleep disorders and epilepsy in order to apply the system to disease models and analyse the impact it can have in the study of disease mechanisms, as well as in the detection of early signs of dysfunction.

The main benefits will be: a) the generation of large, robust datasets obtained in a familiar environment, b) the elimination of experimenter interaction with the mice, which can affect the mouse behaviour during recording, c) the integration of different analysis methods, in the same mouse over a period of time, generating data that can be analysed in depth by many experimenters with different interests, d) a decreased in the number of mice used and e) continuous monitoring in the mouse 24 hours a day, which will mitigate or eliminate welfare issues.

By combining the phenotypic assays we have available, we expect to develop a system that uses fewer animals for each experiment compared to those used in single analysis studies, and at the same time provides more information. In addition to the potential reduction in the number of animals used, we will obtain more data consistency by integrating different readouts from the same animal, leading to a reduction in variability.

These achievements will be disseminated throughout the mouse community and at scientific meetings, so that others may also benefit from the technique.

How will you look to maximise the outputs of this work?

The data collected from this advanced phenotyping will be retained as a permanent digital record, available through a data portal, and we will seek input from other specialists in the field to optimise the technology.

To maximise the outputs of this work, the improved phenotypic analysis will be published in the scientific literature and will be used in successive animal phenotyping studies under the authority of other licences. This new phenotyping system will eventually be extensively applied in the study of our new mouse models and will become part of the tests used for preclinical studies providing more robust and comprehensive data and decreasing the number of mice required.

Species and numbers of animals expected to be used

- Mice: 2670

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This is a technology development licence designed to improve the procedures used to assess phenotypic readouts in mouse models of diseases, where non-animal alternatives are not available or appropriate. As such, it is not possible to use any other species than the mouse for this work.

We will use various strains of adult wild-type mice in order to set up the system, obtain baseline data and refine the analysis. We will also apply the new system to GA mouse strains that model sleep disorders and epilepsy in order to explore its potential in accurately detecting certain physiological states, as well as to potentially develop earlier humane endpoints by using more comprehensive surveillance. Only adult mice will be used for the described experiments.

While developing our phenotyping technology, we will co-house mice in groups of 2-3 in each cage to maintain their welfare and social interactions, and all measurements will be performed in the home cage. Single housing can happen only for a maximum of 10% of the duration of the experiment, for example, when a comparison in the sleep pattern between single-housed and co-housed mice is scientifically necessary.

Typically, what will be done to an animal used in your project?

Typically, an animal will undergo surgery to insert a device for the measurement of electroencephalography (EEG) and electromyografy (EMG) (alone or combined). A device with four wires will be implanted under the skin. Two wires will be inserted within the neck or peripheral muscles to record their activity (EMG). The other two wires will be inserted into the skull to record brain activity (EEG).

During the surgery, animals can also be subcutaneously implanted with a Radio Frequency identification (RFiD) microchip for animal tracking and monitoring.

Following implantation of the device(s), two or three mice will be co-housed in the Home Cage for monitoring activities, behaviour studies and/or food and water intake. Each experiment will use a combination of these tests, which are non-invasive, over the duration of the experiment.

Each recording can be repeated for longitudinal data collection over a maximum of six months. Mice will then be humanely killed or terminally anaesthetised for tissue collection.

What are the expected impacts and/or adverse effects for the animals during your project?

We will use wild-type animals in this study, as well as GA mouse strains that will exhibit sleep dysfunctions and/or epilepsy. Genetic alterations in the mice used in this project may lead to the development of phenotypes such as weight loss, locomotor defects and seizures.

Adverse effects may be encountered due to the anaesthetic and the surgical procedure during the implantation of a telemetry device. Mice will undergo surgery that requires anaesthesia, with a consequent risk of mortality (<5%). Animals are expected to recover from the anaesthetic within 2 hours of the end of the surgical procedure. However, some animals (<5%) may exhibit signs of pain, distress and/or ill health such as a hunched appearance or reduced movement. Weight loss will be monitored daily for 7 days after surgery and mice will need to be culled if the loss is over 15% of the pre-surgery weight (taking into account the weight of the implanted device).

A small percentage of wounds may open (<5%), typically 3-5 days post-surgery. In the majority of such cases, where the wound is clean and dry, local treatment with a topical product may be given, and the wound left to heal.

Pain post-surgery will be controlled by administration of pain reliefs.

In a percentage of animals the recording of the telemetry activity can fail due to misplacement of electrodes (5%) or involuntary contact between the lead wires (10%).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice on the mild breeding protocol are not expected to suffer any adverse effects and the vast majority will not reach the expected severity threshold (90% will have sub-threshold severity).

On the moderate breeding protocol, it is anticipated that any mice carrying the disease-causing phenotype could exhibit a moderate phenotype. Other genotypes will also be born from these crosses, so approximately 25% of the mice may suffer a moderate severity.

Mice on the phenotyping protocols are all expected to reach a moderate severity because they will all undergo surgery. The surgery itself reaches moderate severity as it is invasive and requires the implantation of electrodes/transmitters and animals experience a period of recovery post-surgery.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The primary objective of this project is to develop an improved phenotyping method to refine the current phenotyping technique in mouse models of disease, when non-animal alternatives are not appropriate and/or available. We will develop this new technology by taking advantage of GA lines that model sleep disorders and epilepsy, and that have phenotypes in both electrophysiology and behaviour.

For complex studies, such as electrophysiological recording, behaviour and metabolism, we need to use a mammalian species to understand genetic alterations in the context of the whole organism.

Whilst some preliminary work in some studies could be done in cells or using computer modelling, or in other species, we want researchers to have the availability of the most refined procedure possible when it is necessary to move into mice.

Which non-animal alternatives did you consider for use in this project?

In this licence, we aim to refine existing phenotyping assays used in mouse models. However, we would also consider the developments in the wider field to ensure that, when non-animal alternatives are available, we would take full advantage of them. Where possible we would use data that has already been generated to develop automated techniques, such as image analysis and pattern recognition, and use animals for validation purposes only.

Why were they not suitable?

The aim of this developmental licence is to improve and refine existing procedures for the analysis of GA mouse strains generated by the scientific community. Therefore, the technology we aim to develop is to benefit phenotyping in an entire organism and there are currently no alternatives to the use of mice for this project.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This application is for a technical developmental licence to improve current procedures and to produce a baseline dataset for wild-type and GA mice. Therefore, an exact sample size is difficult to calculate. Current sample sizes are estimated based on previous data.

Mouse lines will only be maintained while there is a justified use for their continued breeding. All breeding schedules will take into account the fecundity and productivity statistics calculated from previous experience of breeding each animal model used. Using such calculations, we will aim to produce cohorts of animals that closely fit the number required for the experiment without excess.

We will use the initial data to plan our pilot experiments. As we will combine two techniques, we expect to reduce the number of mice used in the test while achieving the same statistical power.

While we set up the combination of the two techniques and check for negative signal interactions, we will only use wild-type mice for a pilot experiment and we plan to use three mice for each sex. We will then begin collecting baseline data and will set up the analysis in 3-4 wild-type strains to assess differences between different genetic backgrounds. This will enable us to use further statistical assessment to refine the correct number of animals required for an experiment.

We will then use GA mouse lines that model sleep disorders (up to 4 lines) and epilepsy (up to 4 lines) to validate the protocol and analyse the sensitivity of the combined system to detect mutant phenotypes.

An example of a typical study will use n=12 mice per sex and genotype. Thus, 12 (mice) x 2 (sexes) x 2 (genotypes) = 48 mice. We anticipate undertaking approximately 11 such experiments (4 GA sleep disorder lines, 4 GA epilepsy lines, and 3 wild-type lines for baseline studies).

Mixed-sex groups will be used and 'n' refers to the total number of mice required for one genotype/treatment group for a mixed-sex study.

We would normally use a linear mixed-effects model to analyse these data. This is a statistical model that contains both fixed and random effects and it is particular useful for repeated measurements in studies that extend over a period of time (longitudinal studies). The model allows us to compensate for effects such as sex, batch, date of procedure, cohort, experimenter etc…

To obtain an experimental cohort of 12 mice/sex/genotype we will need 224 mice. We estimate that for the entire project (set up experiment/ baseline data and GA validation) we will need to breed a maximum of 2670 mice.

Pilot experiments for surgical procedures will be carried out in these GA strains to evaluate singular strain differences relating to anaesthesia and recovery.

We expect a <5% risk of death due to the surgery procedure, a 5% risk of lack of signal after telemetry implantation and a 10% risk of signal interference between the telemetry recording and the HCM system recording when simultaneously performed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To reduce the number of animals used, we plan to carry out initial experiments in a subset of wild-type strains to ensure that there is no signal interference between the telemetry system and the HCM system. We will analyse the data from initial pilot studies and for the different strains to recalculate the numbers needed. We will use the minimum number of mice necessary to obtain statistically significant, reproducible and accurate data.

This combined technology has the aim of using less mice than each single experiment. Measurements will be carried out in the same mouse to remove inter-animal variability (i.e. differences between individual mice that can influence the results) and increase the power, thereby decreasing the overall sample size and increasing the scientific utility of the data that is generated. In addition, recordings will be carried out longitudinally over the life-span of the animal, to obtain more accurate data over time, from a smaller number of mice.

We will take advantage of the NC3R's Experimental Design Assistant (EDA) in order to design experiments in the most accurate way, to ensure the use of the minimum number of mice required for the scientific objective and to apply the most appropriate statistical analysis.

Standard Operating Procedures (SOPs) have been written and used routinely for standard tests and will be written for this developmental protocol. This step standardises the way in which the data are collected and reduces the variability, and therefore the sample size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For breeding purposes, we will use the minimum number of mice required for each study and will plan the appropriate number of matings in advance using conventional statistical methodology. Our animal facility staff are skilled in efficient colony management.

Genetically modified lines, which have previously been studied, will be sourced from repositories (these are carefully managed stores in which the DNA of GA mice is stored for future use), to avoid remaking of lines whenever possible. Any excess stock will be offered to other researchers to minimise wastage.

To minimise the number of mice required for surgical procedures, we will establish surgery SOPs and update them as required. We will also ensure that the laboratory staff are highly trained.

Pilot studies will be used to develop the technique and to study system interactions before beginning the experiments in wild-type and GA lines for baseline data collection and analysis.

In addition, we only use the most advanced techniques compatible with the work we do. For example, electrophysiological measurements are recorded using a wireless telemetry system to allow mice to move freely in the cage and the Home Cage Monitoring system has been extensively developed in- house in recent years.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This technology development project aims to develop a more comprehensive phenotyping protocol, to establish telemetry systems and to combine this electrophysiological assessment with the Home Cage Monitoring (HCM) system that provides spatiotemporal data from mice housed in groups of 2 or 3 per cage.

We have chosen to use the mouse for these studies as it is the lowest mammalian species that can be used to provide a full complement of measurable behavioural and physiological parameters .

Wireless telemetry system: we will use this advanced method for electrophysiological recording that allows measurements to be obtained from freely moving mice. This enable longitudinal studies to be performed without intervention from the operator and with less stress for the animals. This telemetry device will allow the measurement of different signals (EEG, EMG or both) and can also allow body temperature recording.

Transponder microchip: RFiD (Radio Frequency identifier) transponders will be inserted into mice to acquire positional data in the Home Cage. The subcutaneous insertion of these transponders will be performed at the same time as the telemetry implantation to minimize stress and discomfort in the mouse.

Home Cage Monitoring: we will obtain large amounts of data using this non-invasive system that measures mice in their home cage. After the initial insertion of the microchip this system does not involve additional pain, suffering or distress for the animals.

There are currently limited methods that can be used for the full characterization of sleep disorders and epilepsy. Both of these disorders require electrophysiological measurement, and behaviour disturbances are very likely to go undetected by normal husbandry or phenotyping methods, as the signs (sleep disturbances and seizures) occur outside of the normal monitoring periods.

The aim of this licence is ultimately to develop systems in which deep characterization of disorders that are difficult to study and manage can be achieved. It is therefore important to use strains in which the clinical need for development is most pertinent and which will be most relevant for future refinement.

The mouse models we propose to use have robust phenotypes and we know from previous studies that these mice will provide consistent, reproducible EEG and EMG data.

In order to set up the system we will need evidence of clinical signs of disease that can be analysed and compared with the phenotype of wild-type mice. Characterization of such variations will help to identify behavioural changes that may occur before, during and after the changes in physiological state.

The comparison of clinical signs and variations in GA mice with those in wild-type mice, will be used to develop the HCM system, with the ultimate aim of reducing or eliminating the use of telemetry and improving animal welfare.

Why can’t you use animals that are less sentient?

This licence has been prepared specifically to develop a new technology for comprehensive phenotyping in mouse models of disease where non-animal alternatives are not suitable or available.

Monitoring post-surgery needs to be carried out in non-anaesthetised mice in order to measure the effectiveness of the behavioural assay and gain information about spatiotemporal movements and muscle activity.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All measurements will be obtained from animals kept in their own home cage without experimental intervention and without any additional stress or discomfort for the mice. Animals will be co-housed in order to maintain their welfare standard. Only for a small percentage of the experimental duration will there be the need, for scientific purposes, to single house the mice. Single-housed mice will be culled after no more than 3 weeks.

The surgery for the implantation of the telemetry device is the only source of harm for the mice. We will carefully follow all necessary steps to minimise the welfare cost for the mice in these procedures.

Surgery is performed in anaesthetised mice with pain killer drugs administered before and after the procedure. The telemetry device and the RiFD microchip will be implanted at the same time to reduce the harm to the mouse and to negate the requirement for a second anaesthetic induction.

Mice will be carefully monitored after the surgery for any adverse effect and will be culled if the effect exceeds the limits that have been established in this licence.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure that the work in this licence is conducted to the highest standards and can be reproduced by other scientists, the ARRIVE guidelines will be followed at all times.

The risk of infection or delayed healing is minimised by following the LASA guidelines on aseptic procedures (LASA 2017 - Guiding Principles for Preparing for and Undertaking Aseptic Surgery).

The animal facility has full AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) and ISO9001-2015 accreditation. To conform to these standards, we work to a high level of quality control on all fronts including husbandry, phenotyping and administrative processes.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed by reviewing the literature, attending scientific meetings and talking with colleagues in the field.

I will be engaged with the NC3Rs website and symposia during the course of this project to remain informed of the last advances in the field.

Any new development that could impact and refine the purpose of this licence will be discussed within the institute and with other experts to get the best advice on how to implement the new findings in our technology.

I am in close contact with all of the suppliers of the equipment that will be used in this study to monitor developments and to obtain support for problem solving.

132. Dopamine Function Related to Behaviour and Models of Schizophrenia and Addiction

Project duration

3 years 0 months

Project purpose

- Basic research

Key words

Addiction, Attention, Dopamine, Motivation, Schizophrenia

Animal types	Life stages
Rats	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this program is to elucidate the role of the neurotransmitter dopamine in the development of psychiatric diseases such as schizophrenia and addiction; to relate these findings to the specific brain regions involved; and to identify potential novel therapeutic drugs to treat these conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Behavioural studies will focus on effects of drugs affecting dopamine function on normal behaviour in rats, and on disrupted behaviours in models of schizophrenia and addiction. Critically, these studies will allow inferences about the mechanisms underlying behavioural

dysfunctions in these diseases, and identifying potential novel medicines for their treatment. Alongside this neurochemical and histological studies in vitro will assess changes in local neural processes in these disease models, linking changes in brain function to abnormal behaviour, and elucidating mechanisms underlying behavioural changes in schizophrenia and addiction. Understanding neurochemical interactions in the brain is vital for elucidating basic mechanisms controlling normal behaviour and highlighting dysfunctions in these specific diseases, which, in turn, are key to developing novel therapeutic strategies for treating these illnesses. Although focused primarily of schizophrenia and addiction, the information gained will also be relevant to understanding dysfunctional processes in other mental illnesses including attention deficit hyperactivity disorder (ADHD) and autism.

What outputs do you think you will see at the end of this project?

The experiments will provide data on (1) changes in fundamental mechanisms controlling dopamine release in local brain areas in models of schizophrenia and addiction; (2) the effects of drugs affecting dopamine function, including potential novel medicines, on cognitive processes, including attention, motivation, learning and memory, which are disturbed in these and other neuropsychiatric diseases; (3) changes in these cognitive processes in rat models of schizophrenia and addiction, and (4) through histological examination post mortem, changes in cellular structure and function in these models.

Results will be disseminated to the scientific community through publication in mainstream scientific journals and presentation at national and international conferences; to undergraduate and postgraduate students in Medicine and Life Sciences through lectures embedded in their respective courses; and to lay audiences through ongoing outreach activities, including lectures to schools and to adult groups.

Who or what will benefit from these outputs, and how?

The results from these studies will give us a better understanding changes in mechanisms controlling dopamine release in key pathways in the brain in models of schizophrenia and addiction, and how this relates to neural mechanisms controlling normal behaviour and dysfunctions which may underlie abnormal processing in these diseases. In the short term these studies will address fundamental neuronal mechanisms controlling dopamine release in the context of normal behaviour, and will provide a better understanding of the origin of psychiatric disease. In the longer term, this knowledge will open up much needed novel therapeutic strategies for the treatment of schizophrenia and addiction.

Measuring changes in histological markers in post mortem tissue will provide information on mechanisms of long term adaptation to drugs, including transmitter mechanisms involved, and how this relates to behavioural changes, helping to identify enduring mechanistic changes which will be relevant to understanding changes occurring in neuropsychiatric disease, thus reinforcing both the short term and long term benefits described above.

In the short and medium term, through dissemination in the scientific literature and at national and international conferences, the results from these behavioural, neurochemical and histological studies will provide benefit to other scientists studying dopamine mechanisms controlling normal and abnormal behaviour, including behaviours associated with schizophrenia, addiction and other psychiatric diseases. At a more general level, principles derived from these studies will also inform other areas of neuroscience, thus providing benefit to scientists beyond the immediate scope of our studies. In the longer term, the development on novel therapeutic strategies derived from our studies will be of benefit to clinicians in providing better drugs to treat these conditions, and to patients who will achieve better control of symptoms with fewer side-effects.

How will you look to maximise the outputs of this work?

Findings will be made available to other scientists through publication in peer-reviewed journals and presentations at scientific conferences and meetings. For example, we have published eight papers, with four more currently being written, from data generated directly from work under the previous project licence (2017 to 2022). In addition, we have presented our findings at national (British Neuroscience Association; British Pharmacological Society) and international (Federation of European Neuroscience Societies; American Society for Neuroscience; International Congress on Monitoring Molecules in Neuroscience) scientific meetings.

Data derived from the work under the licence informs teaching to both undergraduate and postgraduate students. For example, modules on brain and behaviour and on the neuroscience of mental health look in detail at these sorts of approaches for studying normal brain function and dysfunctions in mental disease, with data from these studies used to illustrate points

In addition, the applicant is involved in many outreach activities, presenting talks on brain function to schools and to general public, including activities run as part of Dana Foundation, Brain Awareness Week, where many aspects of brain science are showcased for visitors from schools and from the general public: the applicant has both organised and presented regularly at these events.

Species and numbers of animals expected to be used

- Rats: 600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Studying complex behaviour in the context of changes seen in human neuropsychiatric disease require the use of whole animals. Rats are among the least sentient species which are able to carry out these behavioural tasks reliably, and are therefore our species of choice. Many models of neuropsychiatric disease including schizophrenia and addiction rely on behavioural disruption resembling the human condition, and have been validated for use in rats. Work in our on labs, and elsewhere use very similar behavioural tasks, relying on the same cognitive functions, in humans, enabling relevant translational inferences to be drawn. In addition there is a wealth of experimental data, on behaviour, on neurochemistry and on neurophysiology, both from our labs and elsewhere, which is based on rat studies, facilitating cross-laboratory comparisons, and enabling us to draw conclusions on mechanisms controlling the behaviours.

Given the supposed neurodevelopmental aspects of neuropsychiatric diseases we are studying, particularly changes occurring in adolescence, it is beneficial to look at both adult and juvenile (4 to 6 weeks) rats.

We have carried out some studies using less sentient vertebrates (zebrafish) and invertebrates (planaria), in collaboration with colleagues who are experts in the respective areas. While there has been some success with these studies, their applications to the study of neuropsychiatric disease is limited, due to the differences in complexity and organisation of their nervous systems, compared to mammals.

Typically, what will be done to an animal used in your project?

Workstream 1 : Induction of models of schizophrenia or addiction, in preparation for in vitro or post mortem measurements

1. Animals will be treated over a period of up to 2 weeks (typically one week) with neuroactive drugs (or vehicle) under administration regimes which are known, from previous experiments both in our laboratory and elsewhere, to evoke behavioural changes which resemble changes occurring in schizophrenia or addiction.

2. Typically animals will be left, without further treatment, for at least 7 days to ensure all drugs used in pretreatment have washed out of the system, before further experiments.

3. Animals will be humanely killed either by schedule 1 procedure or, where the tissue is to be used for in vitro measurements, or, where tissue is to be used for post mortem histology, by transcardiac perfusion under terminal anaesthesia, and brain tissue will be taken for ex vivo measurement.

Workstream 2 : Behavioural studies in non pretreated and animals pretreated to induce models of schizophrenia or addiction.

1. Animals may be pretreated over a period of up to 2 weeks (typically one week) with neuroactive drugs (or vehicle) under administration regimes which are known, from

previous experiments both in our laboratory and elsewhere, to evoke behavioural changes which resemble changes occurring in schizophrenia or addiction.

2. Pretreated animals will be left, without further treatment, typically for at least 7 days to ensure all drugs used in pretreatment have washed out of the system, before further experiments.

3. Food or water provision may be restricted.

4. All animals will undergo behavioural testing in a number of procedures known to assess function in a number of different cognitive domains (e.g. attention, motivation, learning, memory). Each animal may be tested on several different behavioural procedures. Behavioural testing may include: presentation of neutral (noise, light) and/or mildly aversive stimuli (loud noise; high frequency noise; weak electric shock).

5. Prior to individual behavioural test sessions, animals may receive systemic (typically intraperitoneal or subcutaneous) injections of neuroactive drugs (or vehicle)

6. After the completion of behavioural assessment, animals will be humanely killed either by schedule 1 procedure or, where the tissue is to be used for in vitro measurements, or, where tissue is to be used for post mortem histology, by transcardiac perfusion under terminal anaesthesia, and brain tissue may be taken for ex vivo measurement.

What are the expected impacts and/or adverse effects for the animals during your project?

Weight loss : We would expect mild weight loss during the course of experiments, as a result of restrictions to food or water. These are likely to be relatively small changes (< 10 %) and are likely only to occur during periods of restriction. In addition, some of the drugs used may cause small weight loss (< 10%), which should be transient, lasting no more than 36 h after drug treatment.

Abnormal behaviour : Some of the neuroactive substances used may cause abnormal behaviour, including hyper- or hypo-locomotion and mild stereotypy. These effects are expected to be mild, and transient, lasting no more than a few hours after drug treatment.

Mild, transient discomfort is expected from aversive stimulation. This may be manifest as hyper- or hypo-locomotion or general agitation, and is expected to last only a few minutes (maximum 60 minutes) after the stimulus presentation.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

In workstream 1, all animals (100%) will be mild, since they will only receive multiple injections (typically intraperitoneal) of drugs or vehicle.

In workstream 2, all animals will be classified as either mild or moderate. Specifically, animals undergoing behavioural testing which includes food or water restriction, and/or presentation of aversive stimuli are classified as moderate (80%), whereas those which only experience behavioural testing which does not include food or water restriction or aversive stimulation are classified as mild (20%).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Due to the nature of the studies on complex behaviour, it is vital to use living, unanaesthetised animals, because we need to measure changes in ongoing behaviour in response to pharmacological and behavioural challenges.

Which non-animal alternatives did you consider for use in this project?

Computer modelling.

We have also considered replacing regulated procedures with non-regulated procedures, by using in vitro preparations and invertebrates.

Why were they not suitable?

Although the data obtained can inform computer models, which are valuable in interpreting the outcomes, such models cannot replace live animals for generating the initial data.

Many of the experiments screening the effects of neuroactive substances, which inform behaviour studies are now carried out in vitro. However such an approach cannot replace the need for live animals for studies of behaviour itself.

Invertebrate models (e.g. planaria) have been used to study relevant drug effects in simple behaviours. However, these animals are not capable of more complex cognitive processes related to neuropsychiatric disease, so whilst they inform our behavioural work in rats, they cannot replace it.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers of animals to be used are determined based on many year of experience in this field of work, which inform power calculations to derive group sizes. Numbers used are kept to the minimum required to achieve scientific endpoints. Experimental procedures will not be repeated, unless justified scientifically, for example for appropriate control conditions, or to ensure previous results from our lab and elsewhere can be reproduced..

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During behavioural testing animals will typically undertake several different behavioural tasks, giving an efficiency of use, and also allowing within subject comparisons across different behavioural domains.

In addition, on completion of the experiments, tissue is taken from the animals for post mortem analysis, maximising the amount of information gained from each animal, and reducing the overall number of animals used.

Experimental design, minimising the numbers of animals used, is also informed by reference to NC3Rs Experimental Design Assistant

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our experimental designs use the minimum numbers of animals required to provide appropriate power in the behavioural tests. In addition, by carrying out multiple behaviours and post mortem analysis on the tissue, we are able to maximise the amount of information obtained from each animals. This approach also allows for powerful within subject analysis across the different parameters measured.

Where the drugs to be tested have measurable effects on dopamine release in brain slices, we now use fast-scan cyclic voltammetry (FSCV) in vitro, rather than the in vivo recording methods previously used, for experiments looking at the basic effects of neuroactive substances on transmitter release. In this way we can pre-screen drugs for follow-up investigation in behaviour tests. Since these in vitro experiments are non-regulated, this has reduced the number of regulated procedures undertaken.

Due to the number of experimental manipulations that can be carried out with our in vitro designs, compared to equivalent methods in vivo, we have also substantially reduced the numbers of animals used overall.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rats have been shown in the past to provide a good animal model of motivational systems, providing the least neurophysiologically sentient species appropriate for these types of study. Many of the studies on which the current research program is based were performed on rats, and therefore a wealth of information exists on which to base theories under scrutiny.

All procedures are the minimum severity level required to produce the required effects. For experiments where we are looking at the effects of chronic drug treatment on subsequent neurotransmitter function, animals receive drug dosing in vivo (mild severity), but then the neurochemical recording is done in vitro. This refinement means that experiments measuring basic neurotransmitter responses to drugs no longer use lengthy, and higher severity, in vivo neurochemical measurement methods.

Where feasible we use behavioural tasks measuring the animal’s innate behaviour (e.g. locomotor activity, novel object recognition), or appetitively motivated tasks (e.g. lever pressing for food, sucrose or water reward). None of these behavioural tests are, in themselves, regulated procedures, but in the latter situation, food or water restriction (regulated procedure) is required in order to ensure that the animal is motivated to perform the behaviour. Experiments where dietary restriction is necessary will routinely use the least severe restriction shown to reliably achieve the required motivation: current experiments are investigating whether we can achieve the required appetitive behaviour without the need for restriction, and if successful, these procedures will be adopted for future experiments. We are also actively working on developing novel tasks (e.g. distraction) which do not require any restriction.

Some experiments require aversive stimulation, since one primary objective of our work is to ascertain what neuronal pathways are involved in processing aversive stimuli. In this case mild footshock is the most effective stimulus, as many published studies (including from our lab) have shown that it provides reproducible effects with only brief transient

stimulation (typically a 1 s train on 6 ms pulses and 40 ms intervals: 25 pulses). The minimum level of stimulation needed to provide a reliable response is used. Ongoing research in our lab is also investigating other aversive stimuli (loud noise or high frequency noise) as an alternative to footshock. Preliminary data from our laboratory suggest that these stimuli are appropriate in some situations, although effects are quite variable: therefore further characterisation is required before they can be adopted routinely.

In all cases procedures are carried out by appropriately trained people.

Why can’t you use animals that are less sentient?

Due to the complex processes underlying the behaviours and neuropsychiatric conditions under scrutiny, less sentient species are not appropriate.

However, we have developed models in zebrafish and in invertebrates (e.g. Planaria flatworms), which are carried out in collaboration with groups from other establishments. We have had some success in developing models of simple behaviours in these species, which can inform our work in rats. However, they are not able to produce the more complex cognitive processes under investigation in this program of work, and so are limited in their application to the current research aims.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Over recent years, we have developed in vitro methods for pre-screening the efficacy of neuroactive substances with potential actions in behavioural tests, and only the most appropriate are taken forward into in vivo (regulated) procedures. We will continue to develop these methods, and implement them in place of in vivo methods where appropriate. However, it is not feasible to use these methods to investigate behaviour, where whole animal, in vivo studies are required.

Within the behavioural experiments, animals are regularly handled, and are acclimatised to behavioural apparatus before testing. This is beneficial both for animal welfare and also for scientific rigour and reproducibility.

We have already introduced refinements in regulated procedures used in behavioural testing.

We have reduced the amount of water or food restriction employed to ensure that animals are motivated to perform the appetitive behaviours under investigation, and will continue to research further refinements in this aspect.

We have also reduced the intensity of aversive stimulation required to the minimum required to achieve reliable scientific endpoints, and we will continue to investigate whether further refinements are feasible.

We keep under review the most appropriate route for drug administration, and have considered oral administration, either in the diet, in the drinking water or by oral gavage. However, (1) simple delivery in the diet or drinking water is not appropriate, as the dose level cannot be controlled and (2) oral administration relies on appropriate absorption of the drug from the gut: this is often poor and/or inconsistent. Therefore oral administration is generally not appropriate for these experiments where precise control of dose is required.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Procedures are guided by information on best practice from NC3Rs, through their website and relevant webinars, via NC3Rs Newsletter and through NC3Rs symposia. In addition, information is regularly circulated from the facility's NIO.

Experimental design is guided by principles outlines in PREPARE, and with reference to NC3Rs research design tool. All practices adhere to Standard Operating Procedures published by the facility, and available from the NIO

All procedures are carried by appropriately trained individuals

I also keep up to date with scientific literature, through which I ensure that I am up to date with methodological advances in the techniques we use, which can improve the welfare of animals whilst maintaining the scientific rigour of the experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will remain informed of advances in 3Rs policy and implementation through local, national and international forums

Information on 3Rs is regularly circulated from the facility's NIO, and via NC3Rs Newsletters. I am also maintain regular direct contact with both, to ensure that I am up to date with relevant information.

I regularly attend, and present at the regional NC3Rs symposium, which is the foremost platform for sharing good practice on 3Rs.

Keeping up to date on scientific literature and attendance at national and international scientific conferences ensures that I remain informed of advances in 3Rs at a national and international level.

133. Hereditary Diseases of the Eye

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Eye, Retina, Genetics, Translation, Therapy

Animal types	Life stages
Mice	adult, juvenile, pregnant, aged, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Genetic eye disease is the leading cause of visual loss in children and adults of working age and remains largely untreatable. This is because, despite an increased understanding of the genetics that underlies these diseases, much less is known about the function of these genes. The aim of this project is to develop mouse models of human eye diseases in order to better understand visual gene function and how it is disrupted in individuals with genetic eye disease, with an ultimate aim of developing treatment strategies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Genetic eye disease is the leading cause of visual loss in children and adults of working age and remains largely untreatable. Whilst gene therapy has given the field hope, improvements in vision following treatment with the only licenced gene therapy are modest. We believe a joint therapeutic approach will be required, where complementary drugs are used alongside to maximise vision improvement and prevent sight loss. This project seeks to develop such drugs.

What outputs do you think you will see at the end of this project?

Firstly, this project will increase our understanding of several aspects of inherited eye disease. Findings will be shared with the scientific community via conference presentations and by publishing our work in high impact, open access scientific journals.

Findings will also be shared with patient communities and the general public by presenting at patient events and public science events.

Secondly, we hope this project will lead to the identification of novel targets that can be translated to therapies for the clinic, thus helping patients with genetic eye disease and easing the economic burden of these diseases.

Who or what will benefit from these outputs, and how?

This project will firstly benefit patients suffering from eye disease by providing them with a better understanding of their condition and potentially leading to new therapeutic strategies to combat their sight loss. Secondly, ocular disorders represent a large financial burden to the NHS and the UK government, so new treatments and better diagnoses based on the research output of this project could alleviate financial spending. Lastly, this project will advance science by increasing our understanding of eye biology,which can be applied to other vertebrate organisms (including humans) and so will be of huge benefit to the scientific community.

How will you look to maximise the outputs of this work?

We already have an extensive list of collaborators, both in the UK and the US, who will maximise the benefits of mice kept on this licence by undertaking experiments that we are not expert in (eg Cryo- electron tomography).

We will maximise the benefits of disseminating our new knowledge by only publishing in open access journals and by presenting our work at the community's biggest eye conferences.

Finally, we strongly believe that negative results should be published, to avoid unnecessary duplication of work, which results in excessive use of animals in research. We plan to do so with the negative results generated in this project.

Species and numbers of animals expected to be used

- Mice: 9000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse retina functions in the same way that occurs in humans and many genetic mutations that cause sight loss in humans also cause sight loss in mice. They are thus excellent models with which to investigate genetic eye disease.

Genetic modification of mice is precise and efficient, allowing quick analysis of a gene/process during eye development and disease.

As we can breed together strains of mice carrying different mutations and tags, we can study complex genetic interactions in biological processes like eye function and make use of our tagged lines across various genetic models.

Further, as mice live mostly in the dark, vision is not their primary sense and so we believe sight loss has less impact on them than other research animals.

Typically, what will be done to an animal used in your project?

On this project licence, we will generate mice that carry mutations that result in visual loss in humans.

Once generated, the mouse lines may well be bred with other mouse lines in order to look at gene- gene interactions.

Mice will be aged for up to 2 years, depending on how quickly their eye disease develops.

Mice will undergo testing to assess vision (optokinetic drum and electrodiagnostic testing) and changes in eye structure (anterior segment imaging, retinal imaging, optical coherence tomography, fluorescein angiography, autofluorescent imaging). Some of these are whilst the mouse is awake (optokinetic drum, anterior segment imaging, retinal imaging), some under general anaesthetic (electrodiagnostic testing, optical coherence tomography, fluorescein angiography, autofluorescent imaging).

Mice may undergo treatment with various drugs, small molecules, fluorescent probes or gene replacement / editing therapies. These may be delivered by mouth (in their food) or by injection.

Mice will eventually be sacrificed and their tissue used for experiments.

What are the expected impacts and/or adverse effects for the animals during your project?

Genetically modified mice may undergo visual loss. Because mice do not rely on their sight to interact with their surroundings, the impact should be minimal and should not affect behaviour.

Mice undergoing tests of visual function or structure will suffer minimal stress if not under a general anaesthetic, but will not have these performed less than one week apart. Most of these assessments will be made under general anesthetic (see above).

Mice undergoing injection of a therapy into their peritoneum will suffer short lived discomfort during the procedure. Mice undergoing injection of a therapy into their eye will be under a general anaesthetic during the procedure.

We do not expect any weight loss or tumour formation for any mice on this project.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

No mice will go beyond a moderate severity.

All mice harbouring mutations that cause visual loss will be mild.

All mice (mutant or wild type) undergoing eye investigation or treatment by injection will be considered moderate.

What will happen to animals at the end of this project?

-
- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The eye is an extremely complex organ with many different cell types that contribute to eye development and function. At present there are no cell culture models that can recapitulate a fully functioning eye. For this reason, animal models are still necessary to study eye function, particularly when assessing the pathways that lead to degenerative

disease. The species of choice for these studies is mice. This is because they are mammals and as such have a retina highly similar to humans and have been proven to display retinal degeneration similar to that observed in humans.

Which non-animal alternatives did you consider for use in this project?

We have previously used human, stem cell-derived retinal organoid models to interrogate genetic eye disease and complement our animal work. Indeed, we will continue to do so. However, a mature, fully formed retina is required when assessing degenerative genetic eye disease, something that stem cells canot offer, and so it is not possible to avoid the use of an animal model.

Why were they not suitable?

Despite advances in retinal organoid technology, it is not yet possible to grow a fully formed retina with an architecture that replicates human retina. Until this technology improves, we will have to use animal models if we are to better understand the changes that occur in genetic eye disease at a whole organ level.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This application for a project licence is following on from a previous mouse licence that allowed us to undertake mouse research on genetic eye disease at our research institute. We have calculated the number of mice used per project and extrapolated for the upcoming 5 years. We predict the number of scientists carrying out animal work to investigate inherited retinal disease at our institute will remain constant over the next 5 years and therefore expect to use a similar number of mice to previous years. The numbers required for upkeep and desired out-breeding of lines are derived from estimates provided by the animal facility. To stay updated on optimized experimental design, we will regularly seek guidance from biostatistical experts employed by our establishments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used the NC3R's Experimental Design Assistant online tool in an attempt to find ways to reduce the number of mice we need to use for this project.

Further, and importantly, we have previously calculated the variability that can occur in our visual function tests. This has allowed us to precisely calculate how many animals we need to carry this test out on to be certain that any difference we see in our mutant mice is real. This means we can use the minimum number of mice whilst still being confident of our results.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Mice have proven to be excellent models for studying visual function so, as such, the techniques used to study the eye in mice have been extensively refined. We are now able to assess degenerative conditions of the eye by imaging the live mouse repeatedly as it ages with machines such as the OCT scanner. This means less mice are required to be killed at different time points to assess changes over time, thus reducing the number of animals used.

The number of animals required for the project will be kept to a minimum through the implementation of good husbandry practice. Furthermore, the number of animals required to confirm statistical significance of any ocular phenotype will be determined using a power calculation before the experiments begin. A statistician will be consulted to ensure that our calculations are correct.

Mouse models will only be generated for genes where no existing mouse models have been generated or the disease-causing potential is already well understood.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The species of choice for these studies is mice. This is because they are mammals and as such have a retina highly similar to humans and have been proven to display retinal degeneration similar to that observed in humans.

The NVS will be consulted prior to all experimentation to ensure that the most appropriate methods to minimise animal suffering. All mice will be closely monitored for signs of distress, and analgesia or euthanasia used as appropriate to minimise animal suffering.

Why can’t you use animals that are less sentient?

We cannot use animals at a more immature life stage because the human disease we are trying to identify treatments for is a degenerative one and therefore the mice only develop signs of disease later in life.

We cannot use a less sentient animal because we need to research an eye that is highly similar to humans, and therefore a mammal is required.

We cannot use an animal that has been terminally anaesthetised because we would be unable to age the mice. Our measurements of retinal structure and function need to be monitored over time, to allow us to determine degeneration. By doing to, we do not need to sacrifice mice at each stage, and therefore are able to reduce the number of mice we use, in keeping with NC3R principles.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Following any procedure, be that an investigation or a treatment, mice will be closely monitored to ensure that the animal is not in pain or distress.

Animals will be monitored frequently for harmful phenotypes, though no adverse effects outside of loss of vision are expected from mice except those associated with normal aging processes, such as skin and eye lesions, rectal prolapse and tumours.

Cages with animals past the age of 12 months will be clearly marked as such and weekly examinations for gross abnormalities in appearance and behaviour performed. Additionally, weight measurements and body condition scoring will be performed and recorded following our Establishment guidelines.

If any adverse symptoms are observed, the NVS and/or NACWO will be immediately informed, and if it is considered that the adverse effect can be swiftly and completely resolved, appropriate action taken in response to advice, such as administration of an analgesic or antibiotic. Otherwise animals will be culled by a schedule 1 method.

The health and welfare of animals being treated with pharmacological agents will be scored using an animal welfare sheet and managed as described above.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All studies conducted under the applicant's license will have satisfied the requirements of the PREPARE guidelines. The applicant will provide the corresponding authors of material generated under the PPL with the information they require to meet the ARRIVE reporting guidelines, or ensure published material is ARRIVE compliant by editing manuscripts themselves.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will constantly be reading the literature and attending conferences, allowing us to discover advances in 3R practices related to eye research and thus refine our project.

134. Production, Breeding & Maintenance of Genetically Altered Zebrafish

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Genetically altered zebra fish, Genes, Breeding, Biomedical research

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is the efficient and standardised production, establishment and archiving of high quality genetically altered Zebrafish and to supply them for research into the study of normal and abnormal physiology, development, disease modelling or biology.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The functions of many genes and their products, either individually or in ways they interact to produce their intended effects, or how they are dysfunctional in disease is currently unknown or not fully understood. To understand better the impact of genes on normal physiological and abnormal disease processes requires, when necessary and justified, the use of genetically altered animals.

What outputs do you think you will see at the end of this project?

The investigations carried out on fish and embryos produced under this licence hope to obtain new knowledge with respect to basic mechanisms of physiology, development and cell biology. Any fish produced or bred under this licence (which may) be used on other Projects will also have publications generated from the research. GA fish, embryos or gametes may be used for invitro work or tissue analysis to provide information that is used to contribute to current or future in vivo projects.

Who or what will benefit from these outputs, and how?

Short term benefits

The short-term benefits from this work include the development of basic understandings of how genetic modifications affect biomedical processes and development.

Long term benefits

We hope that any new information and understandings produced in the work carried out under this licence will lead to new therapies and treatments for a range of diseases and conditions.

How will you look to maximise the outputs of this work?

New information and data from these investigations will be shared to the scientific community in peer- reviewed scientific journals, meetings and seminars. Some end-users take part in sharing knowledge to the wider community through public engagement with relevant charities and organisations.

Species and numbers of animals expected to be used

Zebra fish (Danio rerio): 25000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Zebrafish reproduce easily and quickly, are highly fecund and have morphological and physiological similarities to mammals, and share 70% of the genes found in humans.

All the genetic manipulations will be carried out on embryos immediately after fertilization. Fish >5 days post fertilisation will only be used for natural matings and, on occasion, provision of gametes for IVF or cryopreservation.

Typically, what will be done to an animal used in your project?

The majority of the fish >5 days post fertilisation will only undergo natural matings. Some of these will also have a small part of their caudal fin removed under appropriate analgesia and anaesthesia to establish their genotype. The extraction of gametes may also be undertaken under appropriate analgesia and anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Fish may experience aggressive behaviours of their tanks mates during mating although this is expected to be very low (<2%). Fish needing to have their genotype identified will have a small piece of the caudal fin removed under anaesthetic in order to analyse the tissue, they will also be provided with analgesia, it is expected that this procedure will not cause more than mild or transient distress.

The extraction of gametes will be carried out under anaesthetic and should cause no more than mild or transient distress.

We do not expect fish raised under this licence to have any harmful phenotypes.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Many of the research projects will involve the use of in vitro systems such as cell culture, human tissue assays and computer modelling to complement the animal work. However, in vitro assays cannot adequately model the complete array of molecular, cellular or physiological interactions necessary to fully understand how genetic modifications result in normal or abnormal processes or behaviours.

Which non-animal alternatives did you consider for use in this project?

In vitro systems such as cell culture, human tissue assays and computer modelling.

End-users often use well established cell lines for in vitro work. Applications for the use of a new genetically altered strains (i.e. already established strains but new to the facility) must be approved by the AWERB sub-committee to ensure that all alternatives have been considered.

Why were they not suitable?

In vitro and in silico models cannot completely simulate the complexity of the development and functions of living body systems which will be studied using the animals in this licence.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated from the number of fish used based on the last 5 years with additional to accommodate the increase in the user base over the coming months/years

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All fish breeding will be managed by BSU facility staff to ensure only the required number of fish are bred and maintained in order to supply high quality embryos for research purposes.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

New fish lines will only be introduced into the facility if there is sufficient justification for their use and where there are no non-animal alternatives available. Breeding programmes will be overseen by BSU facility staff to ensure fish do not age beyond 18-24 months

therefore providing fertile and viable fish to ensure a high number of quality embryos are available for research without overbreeding.

Where possible, fish lines will be cryopreserved at the end of research projects to avoid maintaining live animals unnecessarily.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using genetically altered and wild type zebrafish for this project.

With current genome engineering and transposon-based methodologies, it is fairly easy to generate targeted mutations and introduce transgenes in zebrafish. Genome engineering is therefore very efficient with targeting nucleases (10-30% of injected embryos, in our experience). Therefore, genetic engineering at desired locations in the genome can be achieved successfully and with few expected adverse phenotypes resulting.

Fin clipping of live fish under anaesthesia will be used to collect samples for DNA extraction. Pre- and post-procedural Analgesia will be administered for the collection of this tissue

Why can’t you use animals that are less sentient?

All the genetic manipulations will be carried out on embryos immediately after fertilization, and since the fry are under 5 days post fertilization, they do not fall under ASPA protection. Fish >5dpf will only be used for natural matings and, on occassion, provision of gametes for IVF or cryopreservation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The facility has many years' knowledge of breeding and maintaining genetically altered animals. Zebrafish are not expected to have potentially adverse phenotypes however, any mild phenotypes that arise will be ameliorated if possible and carefully monitored.

All fin clipping will be performed on animals old enough and assessed and healthy enough to undergo the procedure.

Where possible and appropriate non-invasive genotyping methods such as swabbing will be used.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We shall follow Laboratory Animal Science Association good practice guidelines for the administration of substances and principles of the Efficient Breeding of Genetically Altered Animals framework published by the Home Office. The NVS will be consulted if there is any uncertainty.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I attend NC3Rs, LASA and IAT webinars and conferences regularly to ensure I am aware of advances in the 3Rs and the facility has an experienced NVS and NACWO who also have up-to-date knowledge of any advances. I am also part of the Animal Welfare and Management Discussion Group and the Home Office Liaison, Training and Information Forum.

135. Maternal Conditioning Effect: Transfer of Volatile from Different Sow Gestation and Lactation Diets to Amniotic Fluid, Milk, Faeces and Carpal Glands

Project duration

2 years 0 months

Project purpose

- Basic research

Key words

Maternal diet, Vertical transfer, Volatiles, Feed intake, Feeding behaviour

Animal types	Life stages
Pigs	adult, pregnant, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this research is to determine whether volatiles from different maternal feed ingredients have different transfer efficiencies into maternal fluids and subsequent effect on feeding behaviour of piglets.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

When piglets are weaned from their sow, they can often have a prolonged period of time where they are reluctant to consume food as it is unfamiliar to them, which is detrimental to piglet health. Poor feed intake immediately after weaning results in gut morphology alterations, inflammation, reduced nutrient absorption and digestion resulting in an

increase in pathogenic bacteria (Moeser et al., 2017). Therefore a key aim in pig production is to maximise feed intake immediately after weaning. By boosting the levels of key aromatic compounds in maternal feed using supplemental volatiles, it is hypothesised that there will be an increased transfer into maternal amniotic fluid and milk, creating feed preferences in piglets that can overcome the reluctance to eat after weaning.

What outputs do you think you will see at the end of this project?

The results of this research could be beneficial in determining whether certain feed ingredients increase volatile transfer to maternal fluids and improve piglet feeding behaviours after weaning. The weaning process can be stressful for pigs and result in prolonged periods of time where they do not eat, this can lead to inflammation in the gut, which can have detrimental effects on the pigs health and weight. By reducing time to first feed and stress associated with unfamiliar feed formats, the health and welfare of pigs could be improved. If these improvements are observed, modification of pig diets to include higher levels of the specific volatiles identified could reduce neophobia in newly weaned piglets and positively alter their feeding behaviour. By understanding this transfer, it could lead to modifications of the maternal diet that would benefit the pig industry worldwide.

Who or what will benefit from these outputs, and how?

The benefit of the outputs will not be realised until all laboratory analyses are conducted after the trial has been complete. Once outputs have been assessed, data and information will be published.

Results obtained from this trial will be beneficial for future researchers to understand which feed ingredients have a higher transfer into maternal fluids and whether different maternal fluids (e.g. amniotic fluid or milk) have different transfer efficiencies. By reducing neophobia in piglets and increasing their feed intake after weaning, it is expected that the health of the piglets will improve compared to piglets who take several hours to eat after weaning. Overall, in the long-term these results could benefit the pig industry worldwide as reduced feed intake after weaning and the subsequent effects on piglet health can be an area of significant economic loss.

How will you look to maximise the outputs of this work?

The results obtained from this study will be published regardless of whether they are the expected outcome. This work is in collaboration with researchers from Australia and therefore outputs will be shared across the pig industry worldwide.

Species and numbers of animals expected to be used

- Pigs: 188

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The species chosen allows for research to be applicable to commercially farmed pigs. The transfer of volatiles specifically happens between sows and their piglets and therefore it is essential to use this stage of pig production for the research.

Typically, what will be done to an animal used in your project?

Sows: 60 sows will be moved into commercial, indoor freedom farrowing crates 5-7 days prior to the expected farrowing date (as part of standard farm practice) and will be fed one of four experimental dies (L1: Low sensory diet; L1+: L1 with volatile booster; L2: high sensory diet; L2+: L2 with volatile booster). Upon entry to the farrowing house, all 60 sows will have blood samples taken from their mammary vein, a swab taken from their carpal gland, a urine sample and a faecal sample will be obtained by inserting two fingers into the rectum to stimulate defecation.

Sows will be induced to increase their likelihood of farrowing during working hours for subsequent sample collection. At farrowing, a colostrum sample will be collected from the first functional anterior teat of 32 sows in total (8 per treatment), between the birth of the 5th and 8th piglet. At day 5 and 12 after birth, from the sows that had samples successfully collected at farrowing, a blood sample will be taken from their mammary vein, a swab taken from their carpal gland, a urine sample and a faecal sample will be obtained by inserting two fingers into the rectum to stimulate defecation. A milk sample will also be collected by separating piglets for one hour prior to the morning feed (sows will still be able to see and hear piglets), the reintroduction of piglets is hoped to stimulate the mammary gland to enable a milk sample to be collected.

Piglets: At farrowing, amniotic fluid will be collected from 32 litters in total, using a collecting tray. From these 32 litters, the umbilical cord from 3 piglets per litter (96 in total) will have a hemostatic clip placed on the loose end and two further clips placed 2-5 cm from the proximal end, the cord will be cut between these clips and the cord attached to the pig will be disinfected. Blood will be collected from the umbilical vein/artery using a needle. From the same piglets, a faecal swab will be taken by inserting a sterile cotton swab into the rectum and rotating, this will be considered the meconium sample.

From the same 3 piglets that were sampled at farrowing, a faecal swab will be collected using a sterile cotton swab inserted into the rectum at 2, 12 and 21 days after birth.

Post-weaning: Approximately 500 pigs from the original 60 sows will be used for the 14 day production trial. During the 14 day trial, the 96 piglets sampled during the pre-weaning stage will be balanced across treatment pens after weaning. The same pigs, with an additional 32 pigs, equalling 128 pigs in total (2 pigs per pen from 64 pens in total) will

have a rectal swab taken, a blood sample collected from the jugular vein and a saliva sample collected by exposing pigs to a sponge for them to chew on, this will be exposed to the pigs prior to sample collection to prevent the novel aspect influencing data obtained. Then 10 days after weaning, a saliva sample will be collected again using a sponge. Finally, at day 14 after weaning, rectal swabs will be taken. At day 14, 6 pigs per treatment (24 in total) will be selected for euthanasia under Schedule 1 of the Animals (Scientific Procedures) Act 1986 and dissection of tissue samples. For the remaining pigs on trial (296), they will be inspected and returned to the commercial herd, this will be the end of the trial.

What are the expected impacts and/or adverse effects for the animals during your project?

Blood sampling: Possible adverse effects include short-term discomfort whilst being restrained (if applicable) as well as at the injection site during and immediately after collection of blood. However, fully trained technicians will complete the sampling and therefore will ensure the animals comfort and minimise stress. Samples will be collected using aseptic techniques. Prior to blood sampling from the sows, an anaesthetic topic cream will be applied to the injection site to minimise discomfort and pain to the sow. All pigs will be monitored to ensure infection of the injection site does not occur, by carrying out daily health checks.

Rectal swabs (sow and piglets): Possible adverse effects include short term discomfort whilst being restrained as well as during the insertion of two fingers (sows) or a sterile cotton swab (piglets/post- wean pigs). However, this reduces the need for a technician to be present in the pen for long periods of time, which can cause stress to the pigs within the pen. Swabs will also be taken by a fully trained technician to minimise stress and discomfort.

Umbilical cord: Possible adverse effects include short term discomfort as the piglet is held while the umbilical cord is clipped and removed. There is the potential for infection to occur at the site of removal, however this will be disinfected upon removal to minimise risk. Blood will only be collected from the umbilical cord once it has been removed. This will be conducted by fully trained technicians and all piglets will be monitored daily to ensure infection of the removal site on the piglet does not occur.

Milk collection: There may be mild levels of stress for sows and their piglets during the separation period prior to milk collection. Piglets will be provided sufficient space and stay within the farrowing pen to ensure the sow can still see and hear her piglets. Sows and her piglets will be closely monitored throughout the separation period to ensure sows do not become higher than mild level of distress, at which point the separation would cease.

Saliva samples- potential stress due to a novel item in the pen, although this will be gradually introduced to reduce that impact for sample collection. Pigs will be able to freely chew on the sponge.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild severity for all sows (60) and piglets/pigs (128) within the trial.

What will happen to animals at the end of this project?

- Kept alive
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The program of work aims to identify the transfer efficiencies of volatiles from sows to piglets and therefore it is not feasible to conduct this form of work in any other animal. Furthermore, given the experimental diets could be beneficial to commercial farming practices worldwide, it is essential to determine their effects on commercially bred and housed pigs.

Which non-animal alternatives did you consider for use in this project?

None

Why were they not suitable?

Given the potential benefit of the sow diets to commercially housed piglets, they are the only appropriate species option and will allow results to be applicable to the industry.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

A sample size has been determined using information gathered from previous published research and similar studies conducted by the researchers involved.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of animals used is the least amount of animals per treatment, as determined by a power calculation, while also allowing for some animals to be removed from the trial if required, without compromising the statistical validity of results. This experiment aims to detect a significant difference (P<0.05) between volatile profile in the amniotic fluid, milk, colostrum, blood, carpal fluid and faeces from sows receiving diets with different volatile profiles. Based on previous research as presented by Val-Laillet et al., 2018 and the use of an online power-sample-size calculator (https://www.gigacalculator.com/calculators/power-sample-sizecalculator.php), with a standardised difference of 0.85, a significance of 0.05 (95% confidence) and 80% power, the minimum sample size required is 8 sows per treatment to find statistically relevant results. Sampling 60 sows at the start of the trial will ensure we can sample from and follow eight sows per treatment (32 in total across both production batches) throughout the farrowing period.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Given the potential difficulty to obtain amniotic and colostrum samples from sows at farrowing, to ensure that litters that prove successful in collection of these samples, all sows will be sampled from at the first time point (entry into the farrowing house). This means that there will not be misalignment in full sample sets over-time. After collection of samples at farrowing, those that were a success, will be the sows/piglets that are sampled from repeatedly, to reduce the total number of animals sampled and to enable accurate result to be determined over time. This includes into the post-weaning stage.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

To determine different transfer efficiencies of volatiles from sow feed to sows and their piglets, analysis (such as gas chromatography and mass spectrometry) of samples from a multitude of fluids will need to be carried out. This will also enable identification of varying transfer efficiencies through different maternal fluids. Volatiles will be measured in sow blood, carpal gland fluid, amniotic fluid, umbilical cord blood, colostrum/milk, urine and faeces. Upon entry to the farrowing house, sow blood, carpal gland fluid, urine and faecal

samples will be collected for analysis to determine volatile levels. Sow faeces will also be used to asses faecal microbiome and compare to that of her piglets. Prior to blood sampling from the sows, an anaesthetic topic cream will be applied to the injection site to minimise discomfort and pain to the sow. Amniotic fluid will be collected from 32 litters using a collection tray.

Piglet umbilical blood and cord will be collected by placing a hemostatic clip on the loose end of the umbilical cord and two further clips at the proximal end, where the cord will be cut between. The remaining cord attached to the piglet will be disinfected and from the cut cord, blood will be extracted using a needle. This will prevent extraction while still attached to the piglet. From the same piglets, a sterile cotton swab will be inserted into the rectum and immediately removed to collect the meconium/faecal samples required.

The same sows and piglets will be sampled from at each time point to reduce the total number of animals sampled. Sampling over time is important to determine whether volatile transfer from feed to sows and her piglets changes over time.

After weaning, the same 96 pigs, with an additional 32, to total 128 pigs (2 pigs per pen) will have a faecal swab, blood sample and saliva sample to determine volatile levels after weaning. The saliva sample will also be used to measure stress levels, to determine whether familiarity with volatiles in the feed reduces stress. Saliva samples will be taken again at day 10 to determine differences in stress response over time and at day 14, faecal swabs will be repeated to determine differences in bacterial composition over time.

Why can’t you use animals that are less sentient?

Given there are direct interactions expected between the sow and her piglets during the gestating and farrowing period, and that the expected results could be beneficial for commercially managed pigs, it would not be appropriate to use any other species than commercially bred and reared pigs.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The regulated procedures involved, will not cause pain, suffering or lasting harm more than mild severity as these procedures will be carried out by fully trained staff that possess a Home Office Personal Licence and up-to-date training. During the entire research trial, all sows and her piglets will be health checked daily to ensure the health of the pigs are maintained. Any piglets showing signs of ill-health, as determined by trained research technicians and/or the veterinarian, will be treated with relevant medication, removed from the trial (if deemed necessary) or euthanized appropriately. A dedicated pig veterinary specialist will be available in these situations. Prior to blood sampling from the sows, an anaesthetic topic cream will be applied to the injection site to minimise discomfort and pain to the sow.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For blood sampling of the mammary vein, the procedures documented in Scollo et al., 2019 will be adhered too: Scollo, A., Bresciani, C., Romano, G., Tagliaferri, L., Righi, F., Parmigiani, E. and Mazzoni, C., 2019. A novel blood-sampling technique in lactating sows: the mammary vein route. The Veterinary Journal, 254, p.105397. As well as the advice regarding volumes presented by Swindle MM (2010). Blood collection in swine.

For blood sampling of pigs during the post-weaning stage of production, the NC3Rs guidance on blood sampling pigs from the external jugular vein (non-surgical) will be used Available at: https://www.nc3rs.org.uk/3rs-resources/blood-sampling/blood-sampling-pig#anchor_4.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Regularly check the NC3Rs website and read the regular emails received as part of the licensee email list from NC3Rs as well as actively looking for advances in the area that could effectively advance the research.

136. Role of Parp7 and Mono-ADP Ribosylation in Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

neuroplasticity, epilepsy, aging, cognitive functions

Animal types	Life stages
Mice	aged, adult, juvenile, neonate, embryo, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

PARP7 is a protein which tags itself and other proteins which activate different mechanisms in the cells. These changes can be induced by normal activities like learning and forming new memories, in the normal aging process, and in diseases like epilepsy. This project aims to investigate how PARP7 is involved in the brain's ability to adapt to changes in stimuli, both in normal health and in disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Neuroplasticity is the ability of the nervous system to change its function and structure in response to its environment. It occurs under normal conditions like when creating a new memory or learning a new task. Excessive neuroplastic changes are a hallmark of epilepsy and its loss is one of the first and most dramatic signs of brain aging. The protein PARP7 adds a specific tag to other proteins in a process known as mono ADP-ribosylation (MARylation). We showed that mice lacking PARP7 have an abnormal brain structure, suggesting that these mice could have difficulties in learning and limited memory. This, and other research, suggests that PARP7 plays a role in neuroplasticity but there is no comprehensive study on it. To gain this deep understanding we will investigate how the loss of PARP7 affects neuroplasticity in different contexts in:

- health - specifically in learning and memory,
- aging - as a model of slow deterioration and loss of neuroplasticity,
- disease - epilepsy, which is characterized by hyperneuroplasticity.

Taken together these results will give a deep understanding of one of the molecular pathways involved and will yield the required knowledge to then be able to manipulate it in diseases such as epilepsy.

Better research into the control and treatment of epilepsy is paramount as there is currently no cure and the consequences of epilepsy are severe and wide-reaching. For example, people with this diagnosis have the lowest rates of employment (34%) and those that work are affected by a pay gap, earning 11.8% less than non-disabled workers.

This project will also test drugs (I.e. RBN-2397) that block this protein in treatment of mouse epileptic brain slices to see if it will reduce the neuronal damage typical of this disease. Because neuroplasticity underpins so many processes and its dysregulation is a hallmark of many neurodegenerative diseases what will be gained by this project can also be applied to other diseases and in health.

What outputs do you think you will see at the end of this project?

- New information
- Publications
- Novel treatment strategy against epilepsy

Who or what will benefit from these outputs, and how?

Asset	Output	Primary beneficiary	Secondary Beneficiary	Timescale
Novel knowledge. Further understanding of physiology, aging and disease	Publications and presentations	Researchers in the field	Wider scientific community	Short term: years 2-5
Open source high throughput data	Raw data deposited in open access repositories	Scientific Community	n/a	Short-term: years 3-5
Novel therapy for epilepsy	Publications and novel use of a drug already in clinical trials for a different disease	Scientific Community	Pharma industry / patients	Mid-long term: from year 5 onwards

How will you look to maximise the outputs of this work?

The outputs of this work will be maximised by:

- disseminating new knowledge through open access publications and pre-print services such as Biorxiv.org

- depositing raw data in open repositories, as to make it available to other researchers

- present the results at national and international conferences

- establish collaborations within the establishment as to allow for the harvest of different tissues from the mice when they are being killed, as to maximise their usability and the data that can be obtained from each mouse. As little is known on the role of PARP7 in different tissues there is scope to use the mice to explore expression and

function on this protein beyond the brain. I have already established various collaborations which will see the isolation of bone marrow, spinal cord, liver and heart.

Species and numbers of animals expected to be used

- Mice: It is anticipated that approximately 450 mice will be used each year

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project relies on genetically modified mouse lines.

Standard tools to image proteins in tissues do not work for PARP7, so we will use a genetically altered mouse line which makes visualizing the protein possible by adding a visual tag to the protein.

These mice will be analysed at all developmental stages from embryonic (10.5 day old embryo) to 18 months as we will look at where and when PARP7 is present in the developing and aging brain.

We will also use other transgenic lines in which the PARP7 protein is rendered non-functional. Using transgenic mice that have a non-functional PARP7 protein is better than temporarily blocking the protein as it causes less stress in the animals as no treatments are required and ensures less variability and off-target effects. This translates in an overall lower number of animals required as there is less variance and the effects observed are definitely PARP7-dependent.

We will use both male and female mice to minimize mouse numbers, wherever applicable.

As this project investigates the role of PARP7 in aging, we will need to house some of the animals for up to 18 months, until they reach old age.

All mice used will be bred at the establishment and the majority (up to 70%) will be killed using the most humane killing methods and tissues taken for ex-vivo studies. Mice will be killed at the optimum age for ex-vivo studies, for most this will be 6-8 weeks but there may be some exceptions.

Animals used in behavioural studies may be assessed at different life stages (juvenile, adult, and aged up to 18 months) to compare how cognitive functions change throughout the animals lifespan.

In some animals epileptic seizures will be induced to allow for the investigation of the role of PARP7 in disease. Epileptic seizures will be done in adult mice (6-8 weeks).

Typically, what will be done to an animal used in your project?

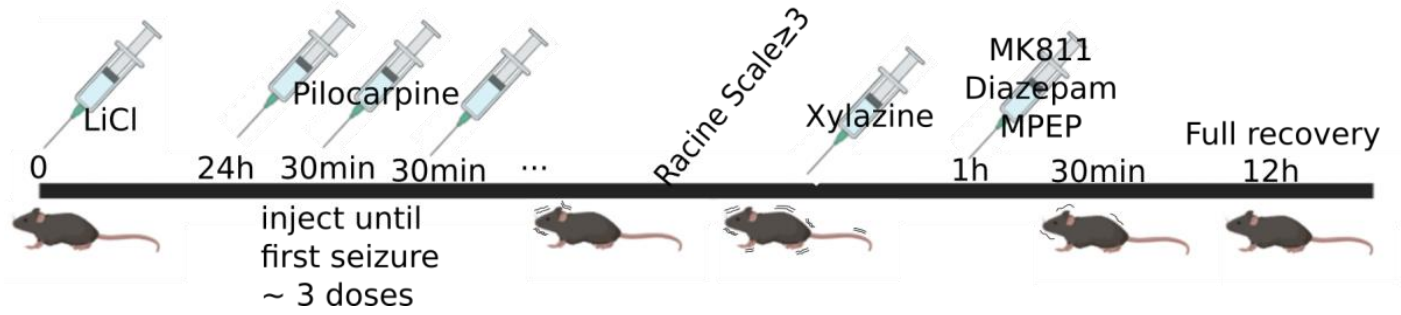
Transgenic mice will be bred at the establishment. These mice will be used for a series of experiments:

They will be subjected to up to 5 behavioural tests for assessment of cognition (1-7) and/or general behaviour patterns may be analysed (8-11) and/or motor coordination (11-13). Animals will undergo a health check in between tests.

1. Radial Arm Maze
2. Object recognition
3. Attentional set-shifting
4. Barnes Maze
5. Y or T maze
6. Morris water maze
7. Hole-board test
8. Irvine Observation Test
9. Open field Test
10. Trace fear conditioning
11. Rotarod
12. Chimney Test
13. Hanging-wire test

These will be repeated at any or all of the life-stages (juvenile, adult, old age) or once following recovery of epileptic seizures. 8-12 animals per experiment per genotype.

- Induction of epileptic seizure: will be done using the most humane and least harmful method which involves injecting a compound to insure seizures (LiCl-Pilocarpine). The mice are then constantly monitored for typical signs of epileptic seizures (in what is known as the Racine Scale), once the animals start to show neck jerking they are injected with other compounds (Xylazine and MK811/Diazepam/MPEP) to ensure reversal of the seizure and ensure recovery:



This procedure will only be done once in each mouse and in about 15-30 mice per genotype will be used.

What are the expected impacts and/or adverse effects for the animals during your project?

- Parp7 mutations and insertions do not cause any adverse effects under normal housing conditions. Females are infertile.

- For the behavioural study slight levels of anxiety can be expected for a short period of time. Fear conditioning tests will cause moderate discomfort.

- When inducing epilepsy the following adverse effects are to be expected: neck jerks, and rhythmic muscular contractions and relaxations of the forelimb muscles - the animals will experience these convulsions for max 1.5hours and at decreasing intensity. Full recovery is expected after 12 hours

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Behavioural tests: moderate severity in the animals that will undergo fear condition testing, which will be 25% of the animals. The other test all have mild severity.

- Epilepsy: severe in a few animals, moderate severity in the majority of animals;

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Due to the complex nature of neuroplasticity which affects how neurons in the brain are connected, determining how the entire organ is wired with different regions working together, non-animal based models are not adequate in addressing the molecular mechanisms. We will also investigate how this affects cognitive functions (memory, anxiety, learning) which cannot be achieved in non-animal models.

Which non-animal alternatives did you consider for use in this project?

In vitro models such as 3D cell cultures are a viable alternative for some aspects of the project and these will indeed be used. An alternative model is a brain organoid. These are artificially grown, in vitro, miniature organs resembling the brain.

Why were they not suitable?

Organoids are not suitable for this project because they are difficult to grow, present great variability across experiments making it difficult to tease the subtle differences characteristic of neuroplastic changes especially in physiological conditions. Furthermore, they are not suitable for behavioural studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The Parp7-/- and the Parp7H532A colonies are bred as heterozygous and the Het:Wt:Ko ratio per litter is approx. 6:3:1 with an average litter size of ~8 pups. Power calculations for the individual experiments showed that a total of 400 (wildtype and mutant) mice will be required. To achieve this, due to the litter size, sex requirements for some experiments and genotype ratio, about 2000 mice have to be bred to have enough mice, according to power calculations, for the planned experiments and for colony maintenance

For the Parp7tm1b colony 200 mice will be needed according to the power calculations.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To reduce the number of animals being used in this project the following steps were taken:

- Experimental design to ensure that animals can be re-used wherever applicable eg combinatorial experiments (behavioural studies under physiological conditions before inducing epilepsy).

- Comparison of different experimental designs to ensure the most appropriate experimental design.

- Use of the NC3Rs Experimental Design Assistant

- Detailed power calculations and statistical predictions based on previous studies were carried out to ensure that the minimal number or animals to achieve statistical significance are used.

- Using PARP7 mutant mice as opposed to inhibiting the enzyme transiently reduces the number of animals used as there are no animals with partial inhibition which would otherwise need to be discarded. It also reduces the number of experiments required to determine that the outcome observed is indeed due to the protein of interest and not by a side-effect.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- Efficient breeding plan which includes planning experiments as to maximise the animals used and intention to use both males and females.

- Sharing of tissues with collaborators and in pilot studies for future projects

- Using organotypic brain cultures that are brain explants cultured and studied in vitro reducing the quantity of animals needed because multiple organotypic slices can be obtained from each brain explant.

- For studies with little or no experimental data available, small pilot studies will be done to determine if there is indeed a difference between the mutant lines and the wildtype

- Wherever applicable, pilot studies using in vitro techniques such as cell culture will be done.

- Taking brains for post-mortem analysis from animals following behavioural testing, we aim to maximise the amount of data collected per animal and therefore reduce the number of animals used in the overall studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

- Using the transgenic mouse line instead of transiently inhibiting the enzyme ensures less distress as the animals are not subjected to extra procedures (e.g. intracranial viral injections)

- When inducing epilepsy we will use recent standardization protocols that have allowed to decrease distress and mortality and minimize discomfort

- In the majority of experiments we will not induce epilepsy in the mice but rather in brain explants, meaning that the animals will not undergo any procedures during their lifetime

- Behavioural studies will be done allowing for appropriate periods of acclitimisation and recovery time between tests. This will decrease stress and anxiety in the animals.

Why can't you use animals that are less sentient?

- Studying animals in old age implies that these reach about 18 months. As we want to observe natural healthy aging we cannot induce early-onset of aging in the animals.

- Terminally anaesthetising the animals will affect the experiments.

- Because the experiments measure behavioural and neurological adaptations less sentient animals are not adequate and would not yield any results.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As previously mentioned, when inducing epilepsy animals will be treated to ensure their full recovery within 12 hours as soon as their seizures reach a given category. Should the seizures not subside 1 hour after treatment the animals will be euthanised. Animals will be continuously monitored until their complete recovery (this is expected to be within 12 hours from the induction of epilepsy).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

- NC3Rs Rodent Models of Epilepsy: https://www.nc3rs.org.uk/rodent-models-epilepsy and the related publication Lidster K, Jefferys JG, Blümcke I et al. (2015). Opportunities for improving animal welfare in rodent models of epilepsy and seizures. Journal of Neuroscience Methods 260: 2-25. doi: 10.1016/j.jneumeth.2015.09.007

● Modebadze T, Morgan NH, Pérès IA, Hadid RD, Amada N, Hill C, Williams C, Stanford IM, Morris CM, Jones RS, Whalley BJ, Woodhall GL. A Low Mortality, High Morbidity Reduced Intensity Status Epilepticus (RISE) Model of Epilepsy and Epileptogenesis in the Rat. PLoS One. 2016 Feb 24;11(2):e0147265. doi: 10.1371/journal.pone.0147265. PMID: 26909803; PMCID: PMC4765932.

● Gouveia, K., Hurst, J. Optimising reliability of mouse performance in behavioural testing: the major role of non-aversive handling. Sci Rep 7, 44999 (2017). https://doi.org/10.1038/srep44999

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

● Subscribing to NC3R's and Norecopa newsletters

● Keeping informed on the latest developments in in vitro models of neuroplasticity via peer- reviewed publications and conferences

These will be implemented in the study by swiftly adapting our protocols and experiments accordingly.

137. Modifying the Early Gut Microbiota to Reduce the Risk of Developing Immune-Associated Disease in Later Life

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Nutritional interventions, Pig model, Microbiota development, Immune development, Metabolic development

Animal types	Life stages
Pigs	neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim is to explore how early nutritional interventions can modify the composition and metabolic output of the gut microbiota using piglets as models for human infants. Early life nutrition is highly likely to influence the development of immune and metabolic systems in via the microbiota, which is linked to how they function in later life. Positive modifications of the gut microbiota in early life could reduce the risk of developing inflammatory, metabolic and autoimmune diseases in later life, which appear to originate very early on.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The gut microbiota is increasingly being linked to various chronic diseases including cardiovascular and liver diseases, as well as autoimmune diseases including type 1 diabetes. There is growing evidence that these may originate during early-life.

Currently, recommendations are to increase dietary protein, but we do not yet know what effect this will have on long term health. Some dietary protein is undigested and ends up in the colon where it is utilized by some gut bacteria and this can skew the microbiota, and the types of metabolites it is producing. This altered microbiota interacts with host immunity and metabolism differently to one which is not fed by excess protein in the diet.

We have previously shown that increased dietary protein could be an important factor in the development of ‘leaky gut’ syndrome where the intestinal barrier does not work as it should. This means that various molecules from food and bacteria cross over into the blood stream and generate low-level immune responses. It is this low-grade inflammation which is known to contribute to later chronic disease.

We also know that female and male guts, immunity, metabolism and microbiotas are very different which suggests they could respond very differently to excess dietary protein and other nutritional interventions. This has important implications when it comes to the development of interventions to reduce leaky gut and therefore later chronic disease.

Although the autoimmune disease type 1 diabetes is diagnosed around 10 years of age, significant changes to the pattern of immune development occur during the first few months of life. Some members of the gut bacteria community produce enzymes which mimic human insulin. If these are present during very early-life, a tolerogenic immune response is generated. However, if they are not presented to the immune system until later on, the immune system can generate an active immune response against them. This can lead to antibody responses against host insulin, which could initiate the development of type ! diabetes. Other microbial products can also mimic host proteins and could be associated with the development of other auto-immune diseases. There is currently no cure for type 1 diabetes and incident rates are increasing. Exposing infant guts to microbially-produced host- protein mimics, such as the insulin mimic, in very early life has potential to prevent the development of autoimmune diseases such, as type 1 diabetes, in high-risk individuals.

This program of work will explore the links between early-life microbiotas and disruption to immune and metabolic development in more detail using piglet models of human infants . It will determine the mechanisms involved using cutting-edge microbiota composition,

metabolic profiling and immunological technologies, and by integrating the data obtained from each of these analytical platforms.

What outputs do you think you will see at the end of this project?

1. Increase understanding of immune and metabolic development in response to increased dietary protein supplementation in a piglet model of human infants.

2. Determination of how that immune and metabolic development is affected by the developing gut bacteria in piglets fed both normal and increased levels of protein in their diets.

3. Determine how the above impacts on intestinal barrier function.

4. Ultimately, demonstrate in an in vivo model that increased dietary protein reduces intestinal barrier function.

Due to our systems approach, this work is highly likely to generate novel information and lead to peer- reviewed publications. It will also generate data to inform the development of further grant applications. Furthermore, considerable physiological similarities between pigs and humans means that findings are more likely to translate into human healthcare than other non-primate models.

Who or what will benefit from these outputs, and how?

Pigs and humans are very similar and so the results we obtain from these studies will benefit humans. Far more so than if this program of work was carried out using mice or rats. The major benefit will be seeing if increased consumption of protein and other nutritional interventions, are good or bad for gut development and subsequent immune and metabolic development. This will be relevant and beneficial for clinicians (especially pediatricians) and infant food development industries.

Many human immunological diseases (autoimmunity, allergy) are being linked to problems with immunological development in early life, but our understanding is limited. Since immune development is driven largely by the gut microbiota, and due to its accessibility for modification, altering the gut microbiota in early -life has the potential to reduce the risk of disease development. This program of work will inform the development of nutritional interventions to reduce the risk of immune-associated diseases in later life. Although this is long term, the benefits of this work could be societal as incidences of immunological-based diseases are increasing rapidly in modern society. There is no cure for many such conditions and so prevention is a valuable approach from which many 'at risk' individuals could benefit in the future.

Academics will benefit from this work as it will help to unravel the complex interactions which occur between the gut microbiota, metabolism and immunity under normal conditions in our control animals. Gut microbiota research is expanding rapidly as it

appears to be involved in many aspects of normal development. This will benefit human and animal medical and nutritional professionals.

Animal feed industries will also benefit from this work. We have already been asked to present finding from these studies at AB Ltd which is a large international animal feed producer (which manufactures our bespoke feed for our trials). We have also received materials in kind from several other animal feed producers and manufacturers which demonstrates their interest in our work and how they may benefit.

In the pig industry, it is essential that piglets 'get off to a good start'. It is likely that information generated by our studies will benefit pig producers. Nutritional intervention in early-life could lead to increases in productivity and welfare within the industry, alth this is a longer-term benefit as we are here proposing fundamental research.

How will you look to maximise the outputs of this work?

Initially, the work proposed will be carried out in collaboration with another university since we have 2 co-supervised PhD students, one based at our institute and the other at the other institute. This work will link with that currently being carried out there by their PhD student which involves human trials and will be informed by this pig work. These parallel studies in pigs and human will be highly informative in terms of understanding universal biological mechanisms underlying links between increased dietary protein and leaky gut across two species. The applicant and the other supervisors all have strong track records of peer-reviewed publications (>200) in a range of journals, and of giving invited talks at international conferences. Taken together, these provide considerable opportunities to disseminate the findings from this work.

In addition, the applicant has a co-supervised PDRA working in a Wellcome Trust funded project in collaboration with medics. As a result, the applicant is often asked to talk at medical conferences, and directly to surgeons and doctors, which also provides opportunity for dissemination of results directly to interested parties.

A further collaboration is being developed with academics at an additional University with whom this program of work has been developed. These academics are renowned researchers in their field and also provide dissemination opportunities. We have already started in vitro work together to inform the development of our pig trials proposed here.

The applicant has up-to-date Loop and ResearchGate profiles (>25k views) and research networks built up over 17 years of working in the field.

In addition, we generate substantial tissue banks from all of our pig trials by collecting an assortment of tissues not necessarily required for the immediate study. We often make tissues and body fluids available upon request to other researchers across the UK and beyond which further maximises outputs from the studies.

Our university has firm commitments to the aims and principles of Open Research and has recently updated its Open Research Action Plan (https://www.reading.ac.uk/research/research- environment/open-research). This promotes the the publication of unsuccessful research approaches in appropriate, publicly available databases.

Species and numbers of animals expected to be used

- Pigs: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

So far, it has not proved possible to model the interaction between gut bacteria and the complex immune, metabolic, nervous and hormone systems of humans without using experimental animals. Less advanced, non-protected, animals do not show the same complex interactions and do not provide reliable models.

Pigs have been chosen for this project because they are one of the best non-primate models for humans. As a consequence, findings will be very relevant to human healthcare. Pigs are especially valuable in early nutrition studies since they can be safely removed from their mothers and therefore environments and nutrition can be very tightly controlled. Animals will be cared for using standard husbandry practices in order to reduce harms and, in addition, their environment will be enriched to permit normal behaviours.

Piglets can be litter matched into treatment groups to reduce the over all number required. Early-life is a critical time for the development of the gut bacteria populations, immune system and metabolic system so it is a good time to assess the impact of dietary intervention on these systems where changes during early life can have lifelong consequences for health.

We have repeatedly demonstrated that apparently minor earlier life events can affect physiological development of immune and metabolic systems. We want to reduce these variables and so only our specific interventions are being measured. If we use older piglets, we cannot guarantee their history, which could easily influence the outcomes of our trials.

In addition, we want to reflect the first few weeks and months of life in infants from as early as possible. It is best to use young piglets for this rather than older animals.

Typically, what will be done to an animal used in your project?

Piglets will be bought to our animal unit, usually (but not always) individually housed and fed a dietary intervention. This will be either standard or high protein diets (for example) and/or commercially available and/or novel probiotics and prebiotics. They will have blood samples taken and faeces will be collected for analysis on a weekly basis. Some piglets may receive standard vaccinations. They will be humanely killed on or before 8 weeks so that other tissues and body fluids can be collected for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

We don't expect to see adverse effects caused by altering nutrition, such as protein levels, in the diet and/or administering probiotics/prebiotics. This is because the levels we will use will be within the normal ranges seen in animal feed and because we have administered prebiotics/probiotics in several trials and seen no adverse effects. Moreover, by definition, prebiotics/probiotics must have a health benefit. We expect to see either no effect, or a benefit.

Piglets will be very young when we transport them to the research site and may have navels that have not completely healed. Infection is a possibility although previous experience tells us that this will be very unlikely. Distress or short-term pain may happen when blood or rectal swab samples are taken.

We expect these effects to be mild and very short-lived. Individually housing piglets can be stressful, but they will be in physical contact with each other and not in visual or contact isolation. At the end of the study, the piglets will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severities experienced by all piglets used in this program of work will be on the lover end of moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

So far, it has not proved possible to model the interaction between gut bacteria and the complex immune, metabolic, nervous and hormone systems of humans without using experimental animals. Less advanced, non-protected, animals do not show the same complex interactions and do not provide reliable models.

Which non-animal alternatives did you consider for use in this project?

We have considered the use of In vitro gut model systems. We use these often as a pre-animal trial screening step, or where we want to assess the effect of, for example, a nutrient (or lack of) on the gut microbiota and/or pathogenic gut bacteria growth and survival. For example, we can screen probiotics/prebiotics for their ability to produce a beneficial change to the gut microbiota and/or its metabolic output prior to selecting specific pre- and probiotics to use in pig trials. This significantly reduces the number of animals we use.

We have completed some in vitro work using gut models in preparation for the first animal trial of this programme of work. We have screened several proteins from different sources (eg soya, milk, egg) for their ability to modify the microbiota using samples from male and female human donors. The preliminary data is promising and demonstrates that we can identify sexually dimorphic differences in metabolite production in response to proteins from different sources. From this work, we have identified a suitable protein, which increases ammonia and phenolic compound production, to take forward into piglet trials.

Why were they not suitable?

Gut models are not suitable for this work since aim is to demonstrate that nutritional intervention has an impact on biological systems, which can not be reflected in vitro. For example, we aim to demonstrate that increased dietary protein has a direct detrimental effect on intestinal barrier function, and that selected probiotics will result in insulin-specific regulatory T-cell populations in the gut mucosa. Neither of these outcomes can be achieved in the absence of an animal model.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have published widely in the area of neonatal nutritional interventions using piglet models and have demonstrated that the numbers proposed are sufficient, but not

excessive, to observe statistical differences in immune and metabolic development in the host.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Professional statisticians were consulted to ensure that experiments in this project were designed using only the number of animals needed for the research questions to be answered.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Litter-matching will be deployed wherever possible. This is where members of the same litter are split between treatment groups to reduce genetic variability between these groups (effectively ‘twin’ studies). Coupled with housing animals in individual units, this strategy means far fewer animals are required than if the treatments were applied to group-housed piglets which had not been litter-matched into treatment groups.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Pigs and humans are very similar and so the results we obtain from these studies will be very relevant to humans. Far more so than if this program of work was carried out using mice or rats. The major benefit will be seeing if increased consumption of protein and other nutritional interventions, are good or bad for gut development. Most other animals are very reliant on their mothers for nurturing and milk when they are born, so their feed cannot be controlled. Pigs are born self-sufficient and so they can easily be taken away from their mothers and given especially formulated feed. This means the experiments are very tightly controlled and the results are very reliable. For these reasons, results from this study will be much better in furthering our understanding of what is occuring at the biological level than if they were carried out in other species. We have only recently become aware of the considerable interactions which occur between the immune and metabolic systems, and that these systems interact closely with the bacteria living in all our guts. However, this is a new area which we do not know much about. We have also become aware that these systems are implicated in several conditions affecting humans, including diabetes,

metabolic syndrome and inflammatory bowel disease. It is important that we understand exactly what is happening at the biological level in order to address these conditions. The proposed studies will go some way to doing this.

This work will use piglet models. This program of work will involve nutritional trials. For example, the level of dietary protein, and proteins from different sources (eg egg, soya, whey) will be used as part of the normal diet fed to piglets. In addition, piglets will be given probiotics and/or prebiotics as part of their diet. These interventions are highly likely to change how the gut becomes colonised with different types of bacteria, and how thesre bacteria behave metabolically. In addition, such changes to the microbiota are highly likely to impact on the early developing immune nsystem, which has been shown to have longer term health implications.

Piglets are very similar to humans in terms of their gut physiology, immunity, metabolism and gut microbiota and therefore are excellent translatable models. The finding from this program of work are more likely to translate into human healthcare than other species might.

These are nuitritional intervention studies and are highly unlikely to cause lasting harm to piglets. There will be some discomfort during bleeding, but this will be very quick and carried out by staff with a lot of experieance bleeding piglets.

Why can’t you use animals that are less sentient?

Less advanced, non-protected, animals do not show the same complex mammalian host-microbe interactions and do not provide reliable models for this sort of work. Data generated using such models is far less likely to translate into human healthcare. The interventions are over several weeks and so using terminally anaesthetized animals is not appropriate.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Trial design will be amended where possible as the program of work progresses. For example where earlier studies in the program of work inform the design of later trials to minimise the welfare costs.

Following each trial, all personnel involved with the project attend a follow up meeting to discuss how the trial progressed, and whether modification could be made to improve welfare during the next trial. For example, following an earlier trial (under a different licence) we concluded that different bedding would be more preferable for the piglets and we have adopted this for subsequent trials. Piglets learn rapidly and we feed them immediately after bleeding with the aim of generating a positive association between feeding and being bled. This may go some way to reduce stress for piglets.

The animal trials do not involve operations and so do not require post-operative care nor pain management.

We will continually review and seek opportunities to refine throughout.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There is currently little specific guidance for neonatal piglet nutrition trials. However, over the years the applicant has developed a network of researchers working in similar areas and we share ideas to promote the refinement of experiments. The applicant has been carrying out piglet trials of a similar nature for over 17 years and has a track record of publishing the results. The applicant was initially trained by experienced pig researchers at a different university who had been working successfully with piglets for several decades under a variety of project licences. Best practices have been shared and this project will follow these best practices and adapt where appropriate to meet specific needs.

Animal staff at our establishment have track records of working with pigs and have completed successful trials in neonatal piglets under the applicant's other project licence (and others). They also regularly complete pig work for other institutes and we often share best practices. For example using a 'bleeding sling' for larger piglets to make the process easier and less stressful for larger piglets.

Specific plans and processes are also discussed with the named vet beforehand and there are several discussions with animal care staff and members of AWERB prior to each pig trial so that the most refined trial can be designed.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The applicant reads the monthly NC3Rs newsletter sent by the institute's UREC secretary. Amendments will be made to the program's animal trials where relevant and appropriate. The PI is also in contact wioth others in the UK working with young piglets and advances in techniques and practices are shared. We have advised other research groups who have since adopted and modified our practisitces to suit their specific purposes.

138. Fish as an Evolutionary Mutant Model to Understand Molecular Mechanisms of Congenital Anomalies

Project duration

5 years 0 months

Project purpose

- Basic research
    - Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

autophagy, neurodegeneration, therapeutics, biomarkers, chaperones

Animal types	Life stages
Mice	adult, juvenile, neonate, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The primary goal of our studies is the development of therapeutics and drugs for neurodegenerative diseases through modulation of cellular pathways important for protein clearance and correct folding.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Proteinopathies are diseases where proteins within cells do not form or function correctly. This includes many of the major neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington's disease. Although these diseases are caused by different types of genetic alterations, they show a common feature in neurons (nerve cells) and their supporting cells: proteins clump into large "aggregated" structures which are toxic and result in neuron death.

There is an urgent need for new drugs and therapies to slow, delay and prevent proteinopathy diseases. This project focuses on two areas that regulate proteins in cells. The first is the cellular processes that enable more rapid removal of proteinopathy proteins, particularly "autophagy" and related pathways that involve autophagy machineries. Autophagy literally means self-eating. The second is chaperone biology. Chaperones are a group of proteins that help other proteins to fold correctly so that they do not clump into aggregates.

Several studies have showed that modulating autophagy or chaperones can be beneficial to treat neurodegenerative diseases and help neurons to cope with protein aggregates.

Moreover, it has also been suggested by several research groups that, when autophagy and related pathways or chaperones function less efficiently in a cell, the nervous system is more prone to develop neurodegenerative diseases.

What outputs do you think you will see at the end of this project?

This work is expected to provide novel information and increase our knowledge about the cellular pathways that regulate protein control in cells in particular protein removal and proper folding. We will continue the work of our previous licence in identifying pathways regulating these processes and drug treatments, which improve the outcomes in Huntington's, Parkinson's and Alzheimer's disease mice.

Our work will be published in peer-reviewed scientific journals and presented to the global scientific community, providing pioneering information for discussion and review.

Who or what will benefit from these outputs, and how?

We are testing approaches and compounds for their therapeutic benefit directly in mouse models of neurodegenerative disease. In the long term, we hope that these studies will be used as the basis for further translational therapeutic trials in human patients. With increased population lifespan, the incidence of people experiencing these devastating disorders will continue to rise. As a result, there is an urgent need for increased

understanding of how these diseases occur and progress, alongside the development of novel therapeutic treatments.

Our work has the potential to impact millions of people worldwide both at the social and economic levels.

The latest figures for dementia alone, showing 55 million people worldwide living with dementia is set to rise to 78 million in 2030 and 139 million in 2050 (Alzheimer's Disease international).

The current total cost of dementia in the UK is £34.7 billion. This is set to rise sharply to £94.1 billion by 2040. These costs is a sum of costs to the NHS, social care costs and costs of unpaid care (Alzheimer's Society UK).

At the second level, the main benefit from work carried out under this license will be scientific advancement, particularly in the areas of protein clearance and chaperone biology. Overall, we hope to identify new genes and compounds that allow us to better understand and manipulate neurodegeneration. This will benefit and guide future therapeutic studies.

How will you look to maximise the outputs of this work?

Results that are not commercially sensitive will be submitted for publication in peer-reviewed scientific journals and presented at international conferences targeting broad scientific communities, in particular.

Successful compound therapeutics will be taken to the clinic, where possible, and unsuccessful treatments shared with the community to prevent others from adopting these approaches. In the longer term, the results produced across this licence regarding the physiology of protein clearance and chaperone biology will guide future approaches to understanding and treating proteinopathies.

Species and numbers of animals expected to be used

- Mice: 20,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our choice of species for this licence is mice. They are the mammalian model of choice for neurodegeneration diseases as genetically modified models exist and have been well characterised, with an increasing pool of mice with engineered genetic changes or containing genes that allow one to monitor specific processes. Mammalian models are

required to provide proof-of principle for therapeutics prior to human studies, and this may be provided with either genetic studies or pharmacological approaches.

We will mainly use adult and aged stage animals to study autophagy and related pathways and their involvement in human disease. In parallel to our work using animals, we will also isolate primary cells, including neurons, glial cells (supporting cells in the brain) and fibroblasts (connective tissue cells). For this reason, we will use mouse embryos or new-born mice.

Typically, what will be done to an animal used in your project?

Over the period of the license, we may generate new mouse lines using standard protocols for superovulation and embryo implantation into pseudo-pregnant females. We will also mate these mice or the mice we already have in house with our mouse models for neurodegeneration or with autophagy reporter mice that produce a fluorescent version of an autophagy protein. These animals will be bred in-house following standard Home Office GAA protocols and maintained by methods appropriate to their genetic alteration, with limited numbers coming from established facilities outside the UK.

We will administer compounds that modulate pathways important for protein clearance and folding through the least invasive method: intraperitoneal (into the cavity of the peritoneum), subcutaneous (under the skin), intravenous injection, food/drinking water, oral gavage (administration via a small tube fed down the throat into the stomach), intranasal (through the nose). However, occasionally we may need to perform surgery to implant a small plastic pump carrying the drug under the mouse skin or deliver the compound into the mouse brain via cannula (a tube) implantation using a three-dimensional coordinate system to locate the specific brain area (stereotactic surgery). Successful compounds will be further tested for their suitability for translation into patients. This will require blood sampling over a period of time to determine the absorption, distribution, metabolism, and elimination of drugs from the body (pharmacokinetic studies), to validate drug effects at concentrations and dosing regimens relevant to those that can be achieved in humans.

In some cases, we will perform food deprivation to induce autophagy. Animals will be maintained on a food deprivation schedule, which will typically occur over two days, and will receive 1.5 hours free access to food per day or sufficient food to maintain body weight at no less than 85% of its maximum body weight. We will ensure all mice get access to food in the feeding period by adding food inside the cage. Body weight will be monitored before and after food deprivation, as well as after the feeding period.

Our approach will include measurement of the levels of the respective defective protein, microscopic examination of tissues, behavioural analysis and ageing studies. To collect and preserve tissues for microscopic examination and biochemical purposes, we may require a non-recovery procedure, such as perfusion fixation (a widely used method that uses the circulatory system to distribute fixatives throughout the body). Moreover, some of

our genetically altered models, such as our autophagy reporter mice that produce a fluorescent version of an autophagy protein, show variable fluorescent protein levels. For this reason, we will may need to perform tail tipping to check for protein levels, as

we have previously found a good correlation with the protein levels in brain. In addition to the microscopy and biochemical studies, we will use behavioural assessments to monitor mouse health and disease progression. The exact set of behavioural tests will be used is dependent on the mouse model being investigated. In each case, a set of behavioural tests will be established that enable the discrimination of the mouse model being studied from the normal animals, whilst causing the least distress to the animals tested.

To further understand their role in autophagy and chaperone modulation in a living organism, we will characterise genetically altered mice for the relevant genes or we will transiently modify gene expression by injecting small interfering RNA molecules or non-pathogenic viral particles both routinely used methods for transiently silencing a gene of interest. These injections will be either done into the tail vein or by stereotactic surgery into the mouse brain.

All mice will be humanely killed at the end of the experimental procedures.

What are the expected impacts and/or adverse effects for the animals during your project?

New mouse lines will may be generated using standard protocols that are expected to result in no more than transient discomfort and no lasting harm.

Genetically altered animals that are mouse models of neurodegeneration show moderate age- dependent disease signs. 50% of mice born will not have the disease gene and are not expected to exhibit any harmful phenotype (characteristics). The remaining 50% carrying the disease gene are expected to have progressive neurodegeneration-related adverse effects, including subdued behaviour patterns even when provoked, hunching and piloerection, tremor, as well as weight loss.

When inducible transgenic systems will be used that allow for the activation of genes in specific cells and tissues at specific times, this will involve the administration of agents to modify transgene expression, an approach that is not expected to result in adverse effects.

When we will administer substances through injections and oral gavage mice (administration via a small tube fed down the throat into the stomach) may experience a mild discomfort, but they are expected to show normal behaviour immediately after the procedure. However, in cases when we have to implant a small plastic pump under the mouse skin or cannula in the mouse brain to deliver drugs this requires surgery and animals will experience short-lived post-operative pain and discomfort. When administrating drugs through pumps under the skin we may need to replace them once (if needed) according to their duration, which varies from 1 to 6 weeks. Although, we

generally use drugs at a safe concentration where no adverse effects are observed, we will closely monitor and if any adverse effects occur we will modify or stop the doses immediately.

Food deprivation is well tolerated in most cases, but it is expected to result in mild weight loss. The total period will not usually exceed 2 successive days but rarely (on a limited number of instances) may be extended to 4 days. Animals will be given a minimum of 1.5 hours free access to food per day or sufficient food to maintain body weight at no less than 85% of its maximum body weight.

Animals that will undergo surgery procedures are expected to have discomfort, therefore peri- and post-operative pain relief will be provided. Animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Mice are also expected to display weight loss.

For the ageing studies, mice will be allowed to approach the end of their natural lives. Although it is not possible to fully predict the adverse effects due to ageing for all animal strains, we expect that it is more likely to develop abdominal tumours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mouse: Sub-threshold 60%

Mouse: Mild 30%

Mouse: Moderate 10%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There is no single alternative to assessing disease models in the context of a mammalian brain, especially if one wants to have a proof-of-principle model for testing potential therapeutics. Assessing genetic and pharmacological changes in the whole animal, the effects on behaviour and relevant outcomes like muscle strength, tremors and balance (depending on the disease) is crucial in order to allow added relevance to the human condition. As such, our cell culture and alternative model approaches guide all elements of our work that cannot be replaced. This includes both aspects of our aims for scientific advancement and therapeutic approaches.

Which non-animal alternatives did you consider for use in this project?

In addition to the wide range of immortalised cell lines we have in the lab, over the period of our last licence, we have extended our use of primary cell culture lines from mice. We routinely culture a broad range of cell lines ranging from mouse embryonic neurons through to embryonic fibroblasts. These allow us to directly address many research questions for functional biology, as well as compound related studies.

We have also recently expanded our cell based assays in neurons derived from human induced pluripotent stem cells (iPSCs), cells that have been derived from somatic cells and that have the

capacity to self-renew and give rise to every other cell type in the body. Also, through collaboration we have access to organoids, self-organized three-dimensional tissue cultures that are derived from human induced pluripotent stem cells (iPSCs).

Why were they not suitable?

Cell based models, while they add support and can help strengthen hypotheses and mechanisms, cannot replace the need for mice, as described above. Cell culture models cannot easily represent long-term behaviour of specific types of neurons through various stages of development. Also, the cell signalling pathways may differ in cultured cells compared to neurons that do not divide. In addition, reversibility of the pathological disease process in an animal could rise from the interaction of different types of neurons and/or with glial cells. Such interactions cannot be fully studied using cell based models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on the studies we are planning to perform, our long-term experience and our current animal usage.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have more than a decade of experience in designing, supervising and performing animal experiments and our experimental design is in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) and PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines. For our studies, we take advantage of the NC3Rs Experimental Design Assistant (EDA) tool.

Moreover, we have a track record of interacting with biostatisticians and we will continue taking their advice on the experimental design and methods of analysis.

We have used power calculations alongside a 15 years of experience when designing experiments. Group sizes to allow sufficient statistical power are dependent on the phenotype we are assessing. We have completed successful studies using sample sizes of approximately 20 animals per treatment group using behavioural approaches to assess disease progression against wild-type or placebo controls. For assessing phenotypes by microscopic examination of tissues smaller groups (generally n=6-10) are required.

We continuously review our studies to assess full results and amend our future plans accordingly.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We breed the minimum number of animals to address our objectives, following the Home Office Advice for standard GA mouse breeding protocols.

When testing new potential drugs in mice with limited information available for the long term use of these drugs in mice, we carry out pilot studies on a small group of non-genetically altered mice (starting with the lowest dose possible and then gradually increase dose) and we will carefully monitor for side effects.

We also maximise our use of animals beyond their lifecycle by routinely collecting and storing tissues for later re-use in other experiments as part of histological and biochemical experiments. This reduces the mice used and in cases where we collaborate with other scientists this reduction is maximised.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All our studies are carefully designed having as top priority animal welfare.

In all our models, we have carefully established processes with monitoring to ensure that we minimise their suffering as a result of the disease or the experimental procedures.

We plan to study at least three mouse models for neurodegenerative diseases including Alzheimer's, Parkinson's and Huntington disease. All these models typically over-express mutant proteins found in patients with familiar types of the respective disease and have age-dependent moderate disease signs. They show clear behavioural, biochemical and tissue changes that we can measure and aim to modify. Moreover, we will study several genetically altered mice to understand the function of autophagy and chaperons in mammals. Some of our mice have an inducible transgenic system that allows for the activation of genes in specific cells and tissues at specific times using agents to modify transgene expression.

When food deprivation will be applied, it will be only for short periods, which in most cases has no major adverse effects other than mild weight loss. Also, we use behavioural assessments to monitor mouse health and disease progression. Our assessment includes both non-regulated and regulated tests, most having no noticeable adverse effects.

When administering drugs, we will use the least invasive method and the majority of mice will not undergo surgery.

Any animal exhibiting signs of pain, distress or of significant ill health will be humanely killed.

Why can't you use animals that are less sentient?

Our project aims in developing therapeutic strategies to treat proteinopathies, the majority of them having a late onset. Accordingly, the neurodegeneration mouse models we study exhibit late-onset symptoms that progress over time, genetically altered animals are initially normal and then develop disease signs with age.

Work completed in the laboratory encompasses cell culture and zebrafish models. Over the last few years, we have substantially increased our efforts using zebrafish. We have been developing new models in the zebrafish that will allow us to use this system either in place of mice, or at least to allow us to refine the experiments that we subsequently carry out in mice. However, there is no single alternative to assessing disease models in the

context of a mammalian brain, especially if one wants to have a proof-of-principle model for testing potential therapeutics.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All our studies are carefully designed, having animal welfare as top priority. In all our models, we have carefully established processes with monitoring to ensure that we minimise their suffering as a result of the disease.

To rapidly and accurately assess the health status of our animals we monitor weight alongside with Body Condition Scoring to define humane endpoints.

It is the aim of all the researchers in the group to communicate efficiently with the technicians who work with our animals. We realise that good communication with them is invaluable - giving them more information about our models and what we expect in each line allows them to inform us quickly and reliably if there is anything we should be aware of concerning the health of our animals. This effective communication allows us to act quickly to reduce the suffering of our animals. Also, we are always seeking advice from collaborators and colleagues regarding approaches that will refine our work with animals.

We will prioritise drugs where pharmacokinetics, brain concentration and dosing are known for mice. When testing drugs with limited information in the literature for their long-term use in mice, we will carry out pilot studies on a small group of non-genetically altered mice (starting with the lowest dose possible and then gradually increase dose), carefully monitoring for side effects. If adverse effects are identified, we may try to decrease drug dose or we will stop doses immediately.

Successful compounds will be tested for their suitability for translation into patients. This will require blood sampling over a period of time to determine the absorption, distribution, metabolism, and elimination of drugs from the body (pharmacokinetic studies), to validate drug effects at concentrations and dosing regimens relevant to those that can be achieved in humans. Compounds with acceptable efficacy in mouse models will be subsequently tested if necessary (e.g. for novel compounds) for safety by assessing whether they have a toxic effect to body systems or organs prior to starting human studies.

It is important to note that constitutive increase in the self-eating pathway (autophagy) is generally beneficial to mouse health and increases lifespan (PMID: 29849149). Furthermore, if we use drugs then the alterations of autophagy will often be effectively transiently allowing return to normality when the drug drops below effective concentrations. For both of these reasons and also because the extensive experience we and others in the field have had testing autophagy inducing strategies in mice, we think that deleterious effects on other organ systems are very unlikely to occur as a consequence of enhancing autophagy.

Some of our studies will require surgical procedures. These are performed under general anaesthesia in aseptic conditions by trained staff. Local pain relief is applied and post-surgery monitoring is also carried out in the recovery room to ensure animals return to normal activity before being returned to normal housing. We favour the administration of pain relief in a flavouring, such as Nutella, which mice will find attractive to eat. This refinement reduces the need to handle animals to administer pain relief by injection. Following surgery and re-housing, they are monitored for activity, weight and wound healing for the next 7 days. We have now started using Nutella and peanut butter in mice that undergo surgery to help them maintain their weight.

In the cases that we will perform food deprivation to induce autophagy we will ensure that all mice get access to food in the feeding period by adding food inside the cage.

When we will use new-born mice to isolate primary cells, we will leave behind 1-2 pups (if in excess) to ensure mother is not getting stressed.

We continuously adapt our approaches to refine our animal studies. Some examples are listed below:

Our Alzheimer's/tauopathy model shows a tendency to be hyperactive, a phenotype which is characterised by them running inside the cage. We noticed that this behaviour was occasionally causing the bedding to become tangled around the leg of the animal causing injury. We therefore changed the type of bedding used for softer material, which has prevented this injury.

Our Parkinson's model shows hyperactivity when provoked, a phenotype which is characterised by them jumping or even having seizures upon disruption of the cage. We have noticed that this behaviour is improved when working in red light, we are therefore using the red light whenever possible.

In some of our studies, we deliver drugs via an implanted small plastic pump under the mouse skin. We have observed that soaking these pumps in saline overnight prior to implantation reduces adhesions formed with the skin of the animals and have therefore adopted this as standard practice. Whenever suitable for our studies, we will favour the use of plastic pumps that can be refilled without the need of anaesthesia and therefore minimising animal suffer.

We also deliver compounds via oral gavage (administration via a small tube fed down the throat into the stomach) - in this process we use flexible tubing for administration. Moreover, we dip the tip in a sweet solution before starting the procedure to make it more palatable for the mice to swallow.

We have started performing stereotactic surgeries using a three-dimensional coordinate system that enables us to study transient effects vs the long-term effect of gene

modification (e.g. in a transgenic model) in the mouse brain - this may reduce possible adverse effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For our study design, we follow the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) and ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and we take advantage of the NC3Rs Experimental Design Assistant (EDA) tool.

For all our experiments involving surgery, we follow the "Guiding Principles for Preparing for and Undertaking Aseptic Surgery, 2nd Edition April 2017".

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are always seeking advice from collaborators, colleagues and the staff at the animal facility regarding approaches that will refine our work with animals and will have a positive impact in the 3Rs.

We also refer to the latest practical guidance from Laboratory Animal Science Association (LASA) that provides recommendations and advances in animal techniques.

We participate in the User's meetings hold regularly by the animal facility and we are members of the 3Rs enquiry list. Therefore, we get informed of any advances in the 3Rs.

139. Understanding the Roles of Autophagy and Related Pathways in Proteinopathies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

autophagy, neurodegeneration, therapeutics, biomarkers, chaperones

Animal types	Life stages
Mice	adult, juvenile, neonate, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The primary goal of our studies is the development of therapeutics and drugs for neurodegenerative diseases through modulation of cellular pathways important for protein clearance and correct folding.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Proteinopathies are diseases where proteins within cells do not form or function correctly. This includes many of the major neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington's disease. Although these diseases are caused by different types of genetic alterations, they show a common feature in neurons (nerve cells) and their supporting cells: proteins clump into large "aggregated" structures which are toxic and result in neuron death.

There is an urgent need for new drugs and therapies to slow, delay and prevent proteinopathy diseases. This project focuses on two areas that regulate proteins in cells. The first is the cellular processes that enable more rapid removal of proteinopathy proteins, particularly "autophagy" and related pathways that involve autophagy machineries. Autophagy literally means self-eating. The second is chaperone biology. Chaperones are a group of proteins that help other proteins to fold correctly so that they do not clump into aggregates.

Several studies have showed that modulating autophagy or chaperones can be beneficial to treat neurodegenerative diseases and help neurons to cope with protein aggregates.

Moreover, it has also been suggested by several research groups that, when autophagy and related pathways or chaperones function less efficiently in a cell, the nervous system is more prone to develop neurodegenerative diseases.

What outputs do you think you will see at the end of this project?

This work is expected to provide novel information and increase our knowledge about the cellular pathways that regulate protein control in cells in particular protein removal and proper folding. We will continue the work of our previous licence in identifying pathways regulating these processes and drug treatments, which improve the outcomes in Huntington's, Parkinson's and Alzheimer's disease mice.

Our work will be published in peer-reviewed scientific journals and presented to the global scientific community, providing pioneering information for discussion and review.

Who or what will benefit from these outputs, and how?

We are testing approaches and compounds for their therapeutic benefit directly in mouse models of neurodegenerative disease. In the long term, we hope that these studies will be used as the basis for further translational therapeutic trials in human patients. With increased population lifespan, the incidence of people experiencing these devastating disorders will continue to rise. As a result, there is an urgent need for increased understanding of how these diseases occur and progress, alongside the development of novel therapeutic treatments.

Our work has the potential to impact millions of people worldwide both at the social and economic levels.

The latest figures for dementia alone, showing 55 million people worldwide living with dementia is set to rise to 78 million in 2030 and 139 million in 2050 (Alzheimer's Disease international).

The current total cost of dementia in the UK is £34.7 billion. This is set to rise sharply to £94.1 billion by 2040. These costs is a sum of costs to the NHS, social care costs and costs of unpaid care (Alzheimer's Society UK).

At the second level, the main benefit from work carried out under this license will be scientific advancement, particularly in the areas of protein clearance and chaperone biology. Overall, we hope to identify new genes and compounds that allow us to better understand and manipulate neurodegeneration. This will benefit and guide future therapeutic studies.

How will you look to maximise the outputs of this work?

Results that are not commercially sensitive will be submitted for publication in peer-reviewed scientific journals and presented at international conferences targeting broad scientific communities, in particular.

Successful compound therapeutics will be taken to the clinic, where possible, and unsuccessful treatments shared with the community to prevent others from adopting these approaches. In the longer term, the results produced across this licence regarding the physiology of protein clearance and chaperone biology will guide future approaches to understanding and treating proteinopathies.

Species and numbers of animals expected to be used

- Mice: 20,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our choice of species for this licence is mice. They are the mammalian model of choice for neurodegeneration diseases as genetically modified models exist and have been well characterised, with an increasing pool of mice with engineered genetic changes or containing genes that allow one to monitor specific processes. Mammalian models are required to provide proof-of principle for therapeutics prior to human studies, and this may be provided with either genetic studies or pharmacological approaches.

We will mainly use adult and aged stage animals to study autophagy and related pathways and their involvement in human disease. In parallel to our work using animals, we will also isolate primary cells, including neurons, glial cells (supporting cells in the brain) and fibroblasts (connective tissue cells). For this reason, we will use mouse embryos or new-born mice.

Typically, what will be done to an animal used in your project?

Over the period of the license, we may generate new mouse lines using standard protocols for superovulation and embryo implantation into pseudo-pregnant females. We will also mate these mice or the mice we already have in house with our mouse models for neurodegeneration or with autophagy reporter mice that produce a fluorescent version of an autophagy protein. These animals will be bred in-house following standard Home Office GAA protocols and maintained by methods appropriate to their genetic alteration, with limited numbers coming from established facilities outside the UK.

We will administer compounds that modulate pathways important for protein clearance and folding through the least invasive method: intraperitoneal (into the cavity of the peritoneum), subcutaneous (under the skin), intravenous injection, food/drinking water, oral gavage (administration via a small tube fed down the throat into the stomach), intranasal (through the nose). However, occasionally we may need to perform surgery to implant a small plastic pump carrying the drug under the mouse skin or deliver the compound into the mouse brain via cannula (a tube) implantation using a three-dimensional coordinate system to locate the specific brain area (stereotactic surgery). Successful compounds will be further tested for their suitability for translation into patients. This will require blood sampling over a period of time to determine the absorption, distribution, metabolism, and elimination of drugs from the body (pharmacokinetic studies), to validate drug effects at concentrations and dosing regimens relevant to those that can be achieved in humans.

In some cases, we will perform food deprivation to induce autophagy. Animals will be maintained on a food deprivation schedule, which will typically occur over two days, and will receive 1.5 hours free access to food per day or sufficient food to maintain body weight at no less than 85% of its maximum body weight. We will ensure all mice get access to food in the feeding period by adding food inside the cage. Body weight will be monitored before and after food deprivation, as well as after the feeding period.

Our approach will include measurement of the levels of the respective defective protein, microscopic examination of tissues, behavioural analysis and ageing studies. To collect and preserve tissues for microscopic examination and biochemical purposes, we may require a non-recovery procedure, such as perfusion fixation (a widely used method that uses the circulatory system to distribute fixatives throughout the body). Moreover, some of our genetically altered models, such as our autophagy reporter mice that produce a fluorescent version of an autophagy protein, show variable fluorescent protein levels. For

this reason, we will may need to perform tail tipping to check for protein levels, as we have previously found a good correlation with the protein levels in brain. In addition to the microscopy and biochemical studies, we will use behavioural assessments to monitor mouse health and disease progression. The exact set of behavioural tests will be used is dependent on the mouse model being investigated. In each case, a set of behavioural tests will be established that enable the discrimination of the mouse model being studied from the normal animals, whilst causing the least distress to the animals tested.

To further understand their role in autophagy and chaperone modulation in a living organism, we will characterise genetically altered mice for the relevant genes or we will transiently modify gene expression by injecting small interfering RNA molecules or non-pathogenic viral particles both routinely used methods for transiently silencing a gene of interest. These injections will be either done into the tail vein or by stereotactic surgery into the mouse brain.

All mice will be humanely killed at the end of the experimental procedures.

What are the expected impacts and/or adverse effects for the animals during your project?

New mouse lines will may be generated using standard protocols that are expected to result in no more than transient discomfort and no lasting harm.

Genetically altered animals that are mouse models of neurodegeneration show moderate age- dependent disease signs. 50% of mice born will not have the disease gene and are not expected to exhibit any harmful phenotype (characteristics). The remaining 50% carrying the disease gene are expected to have progressive neurodegeneration-related adverse effects, including subdued behaviour patterns even when provoked, hunching and piloerection, tremor, as well as weight loss.

When inducible transgenic systems will be used that allow for the activation of genes in specific cells and tissues at specific times, this will involve the administration of agents to modify transgene expression, an approach that is not expected to result in adverse effects.

When we will administer substances through injections and oral gavage mice (administration via a small tube fed down the throat into the stomach) may experience a mild discomfort, but they are expected to show normal behaviour immediately after the procedure. However, in cases when we have to implant a small plastic pump under the mouse skin or cannula in the mouse brain to deliver drugs this requires surgery and animals will experience short-lived post-operative pain and discomfort. When administrating drugs through pumps under the skin we may need to replace them once (if needed) according to their duration, which varies from 1 to 6 weeks. Although, we generally use drugs at a safe concentration where no adverse effects are observed, we will closely monitor and if any adverse effects occur we will modify or stop the doses immediately.

Food deprivation is well tolerated in most cases, but it is expected to result in mild weight loss. The total period will not usually exceed 2 successive days but rarely (on a limited number of instances) may be extended to 4 days. Animals will be given a minimum of 1.5 hours free access to food per day or sufficient food to maintain body weight at no less than 85% of its maximum body weight.

Animals that will undergo surgery procedures are expected to have discomfort, therefore peri- and post-operative pain relief will be provided. Animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Mice are also expected to display weight loss.

For the ageing studies, mice will be allowed to approach the end of their natural lives. Although it is not possible to fully predict the adverse effects due to ageing for all animal strains, we expect that it is more likely to develop abdominal tumours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mouse: Sub-threshold 60%
- Mouse: Mild 30%
- Mouse: Moderate 10%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There is no single alternative to assessing disease models in the context of a mammalian brain, especially if one wants to have a proof-of-principle model for testing potential therapeutics. Assessing genetic and pharmacological changes in the whole animal, the effects on behaviour and relevant outcomes like muscle strength, tremors and balance (depending on the disease) is crucial in order to allow added relevance to the human condition. As such, our cell culture and alternative model approaches guide all elements of our work that cannot be replaced. This includes both aspects of our aims for scientific advancement and therapeutic approaches.

Which non-animal alternatives did you consider for use in this project?

In addition to the wide range of immortalised cell lines we have in the lab, over the period of our last licence, we have extended our use of primary cell culture lines from mice. We routinely culture a broad range of cell lines ranging from mouse embryonic neurons through to embryonic fibroblasts. These allow us to directly address many research questions for functional biology, as well as compound related studies.

We have also recently expanded our cell based assays in neurons derived from human induced pluripotent stem cells (iPSCs), cells that have been derived from somatic cells and that have the capacity to self-renew and give rise to every other cell type in the body. Also, through collaboration we have access to organoids, self-organized three-dimensional tissue cultures that are derived from human induced pluripotent stem cells (iPSCs).

Why were they not suitable?

Cell based models, while they add support and can help strengthen hypotheses and mechanisms, cannot replace the need for mice, as described above. Cell culture models cannot easily represent long-term behaviour of specific types of neurons through various stages of development. Also, the cell signalling pathways may differ in cultured cells compared to neurons that do not divide. In addition, reversibility of the pathological disease process in an animal could rise from the interaction of different types of neurons and/or with glial cells. Such interactions cannot be fully studied using cell based models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on the studies we are planning to perform, our long-term experience and our current animal usage.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have more than a decade of experience in designing, supervising and performing animal experiments and our experimental design is in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) and PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines. For our studies, we take advantage of the NC3Rs Experimental Design Assistant (EDA) tool.

Moreover, we have a track record of interacting with biostatisticians and we will continue taking their advice on the experimental design and methods of analysis.

We have used power calculations alongside a 15 years of experience when designing experiments. Group sizes to allow sufficient statistical power are dependent on the phenotype we are assessing. We have completed successful studies using sample sizes of approximately 20 animals per treatment group using behavioural approaches to assess disease progression against wild-type or placebo controls. For assessing phenotypes by microscopic examination of tissues smaller groups (generally n=6-10) are required.

We continuously review our studies to assess full results and amend our future plans accordingly.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We breed the minimum number of animals to address our objectives, following the Home Office Advice for standard GA mouse breeding protocols.

When testing new potential drugs in mice with limited information available for the long term use of these drugs in mice, we carry out pilot studies on a small group of non-genetically altered mice (starting with the lowest dose possible and then gradually increase dose) and we will carefully monitor for side effects.

We also maximise our use of animals beyond their lifecycle by routinely collecting and storing tissues for later re-use in other experiments as part of histological and biochemical experiments. This reduces the mice used and in cases where we collaborate with other scientists this reduction is maximised.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All our studies are carefully designed having as top priority animal welfare.

In all our models, we have carefully established processes with monitoring to ensure that we minimise their suffering as a result of the disease or the experimental procedures.

We plan to study at least three mouse models for neurodegenerative diseases including Alzheimer's, Parkinson's and Huntington disease. All these models typically over-express mutant proteins found in patients with familiar types of the respective disease and have age-dependent moderate disease signs. They show clear behavioural, biochemical and tissue changes that we can measure and aim to modify. Moreover, we will study several genetically altered mice to understand the function of autophagy and chaperons in mammals. Some of our mice have an inducible transgenic system that allows for the activation of genes in specific cells and tissues at specific times using agents to modify transgene expression.

When food deprivation will be applied, it will be only for short periods, which in most cases has no major adverse effects other than mild weight loss. Also, we use behavioural assessments to monitor mouse health and disease progression. Our assessment includes both non-regulated and regulated tests, most having no noticeable adverse effects.

When administering drugs, we will use the least invasive method and the majority of mice will not undergo surgery.

Any animal exhibiting signs of pain, distress or of significant ill health will be humanely killed.

Why can't you use animals that are less sentient?

Our project aims in developing therapeutic strategies to treat proteinopathies, the majority of them having a late onset. Accordingly, the neurodegeneration mouse models we study exhibit late-onset symptoms that progress over time, genetically altered animals are initially normal and then develop disease signs with age.

Work completed in the laboratory encompasses cell culture and zebrafish models. Over the last few years, we have substantially increased our efforts using zebrafish. We have been developing new models in the zebrafish that will allow us to use this system either in place of mice, or at least to allow us to refine the experiments that we subsequently carry out in mice. However, there is no single alternative to assessing disease models in the context of a mammalian brain, especially if one wants to have a proof-of-principle model for testing potential therapeutics.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All our studies are carefully designed, having animal welfare as top priority. In all our models, we have carefully established processes with monitoring to ensure that we minimise their suffering as a result of the disease.

To rapidly and accurately assess the health status of our animals we monitor weight alongside with Body Condition Scoring to define humane endpoints.

It is the aim of all the researchers in the group to communicate efficiently with the technicians who work with our animals. We realise that good communication with them is invaluable - giving them more information about our models and what we expect in each line allows them to inform us quickly and reliably if there is anything we should be aware of concerning the health of our animals. This effective communication allows us to act quickly to reduce the suffering of our animals. Also, we are always seeking advice from collaborators and colleagues regarding approaches that will refine our work with animals.

We will prioritise drugs where pharmacokinetics, brain concentration and dosing are known for mice. When testing drugs with limited information in the literature for their long-term use in mice, we will carry out pilot studies on a small group of non-genetically altered mice (starting with the lowest dose possible and then gradually increase dose), carefully monitoring for side effects. If adverse effects are identified, we may try to decrease drug dose or we will stop doses immediately.

Successful compounds will be tested for their suitability for translation into patients. This will require blood sampling over a period of time to determine the absorption, distribution, metabolism, and elimination of drugs from the body (pharmacokinetic studies), to validate drug effects at concentrations and dosing regimens relevant to those that can be achieved in humans. Compounds with acceptable efficacy in mouse models will be subsequently tested if necessary (e.g. for novel compounds) for safety by assessing whether they have a toxic effect to body systems or organs prior to starting human studies.

It is important to note that constitutive increase in the self-eating pathway (autophagy) is generally beneficial to mouse health and increases lifespan (PMID: 29849149). Furthermore, if we use drugs then the alterations of autophagy will often be effectively transiently allowing return to normality when the drug drops below effective concentrations. For both of these reasons and also because the extensive experience we and others in the field have had testing autophagy inducing strategies in mice, we think that deleterious effects on other organ systems are very unlikely to occur as a consequence of enhancing autophagy.

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In the cases that we will perform food deprivation to induce autophagy we will ensure that all mice get access to food in the feeding period by adding food inside the cage.

When we will use new-born mice to isolate primary cells, we will leave behind 1-2 pups (if in excess) to ensure mother is not getting stressed.

We continuously adapt our approaches to refine our animal studies. Some examples are listed below:

Our Alzheimer's/tauopathy model shows a tendency to be hyperactive, a phenotype which is characterised by them running inside the cage. We noticed that this behaviour was occasionally causing the bedding to become tangled around the leg of the animal causing injury. We therefore changed the type of bedding used for softer material, which has prevented this injury.

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In some of our studies, we deliver drugs via an implanted small plastic pump under the mouse skin. We have observed that soaking these pumps in saline overnight prior to implantation reduces adhesions formed with the skin of the animals and have therefore adopted this as standard practice. Whenever suitable for our studies, we will favour the use of plastic pumps that can be refilled without the need of anaesthesia and therefore minimising animal suffer.

We also deliver compounds via oral gavage (administration via a small tube fed down the throat into the stomach) - in this process we use flexible tubing for administration. Moreover, we dip the tip in a sweet solution before starting the procedure to make it more palatable for the mice to swallow.

We have started performing stereotactic surgeries using a three-dimensional coordinate system that enables us to study transient effects vs the long-term effect of gene modification (e.g. in a transgenic model) in the mouse brain - this may reduce possible adverse effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For our study design, we follow the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) and ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and we take advantage of the NC3Rs Experimental Design Assistant (EDA) tool.

For all our experiments involving surgery, we follow the "Guiding Principles for Preparing for and Undertaking Aseptic Surgery, 2nd Edition April 2017".

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are always seeking advice from collaborators, colleagues and the staff at the animal facility regarding approaches that will refine our work with animals and will have a positive impact in the 3Rs.

We also refer to the latest practical guidance from Laboratory Animal Science Association (LASA) that provides recommendations and advances in animal techniques.

We participate in the User's meetings hold regularly by the animal facility and we are members of the 3Rs enquiry list. Therefore, we get informed of any advances in the 3Rs.

140. Development of Novel Epitope-Focused Vaccines by Computational Approaches

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Vaccination, Pre-clinical, Antigen, Communicable diseases

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop and test next-generation vaccines in a pre-clinical rodent model.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Communicable diseases cause a huge health burden each year, contributing substantially to global death and disability. This burden is particularly felt by low and lower-middle-income countries. Indeed, the World Health Organisation (WHO) set out in 2015 in its “From MDGs to SDGs” report (where SDG stands for Sustainable Development Goal and MDG stands for Millennium Development Goal), specifically to focus global attention on ending epidemics such as HIV, tuberculosis, malaria, hepatitis and neglected tropical diseases [1]. Such communicable diseases typically result in morbidity and/or mortality, reducing the years of healthy life as measured by Disability Adjusted Life Years. In addition, such diseases also have a large economic impact, further contributing to global income inequality. The COVID-19 pandemic in particular has highlighted the health and economic impact of communicable diseases.

In the case of malaria, an estimated 409,000 deaths occurred in 2019, down from a previous 736,000 in 2000 [2]. However, due to the increasing prevalence of drug resistance, this progress is under threat. Unfortunately, the case is similar for many other diseases where drugs are used to control symptoms, progression or spread. This includes tuberculosis; one of the leading causes of death worldwide. In the case of tuberculosis, the COVID-19 pandemic has regressed progress made in recent years by reducing diagnosis and access to treatment, including specific treatment for drug resistant strains, potentially enhancing drug resistance [3]. In order to tackle such diseases, it is clear that effective vaccination is required. Indeed, vaccination is the only method by which a disease has ever been eradicated; the World Health Organisation declared that smallpox had been eradicated in 1980. By vaccinating individuals, disease severity can be eliminated or greatly reduced, and transmission limited.

In addition to communicable diseases in humans, transmissible veterinary diseases also have a substantial economic and environmental impact. Diseases such as African swine fever; that impacts both domestic and wild pigs, can cause devastation when entire herds of animals must be culled in order to prevent the spread of disease. Current disease control measures include tight biosecurity and import measures in areas unaffected by such diseases, strict sanitation procedures and humane killing of infected animals [4]. An effective vaccine for such diseases would not only alleviate suffering in livestock, but also likely have a positive economic effect, particularly in low and lower-middle-income countries.

Unfortunately, to date, many vaccines have failed or under-achieved due to pathogen complexity or the rate of pathogen mutation. Taking the case of malaria, the vaccine RTS,S has recently been licensed for broad use. However, this vaccine focuses on one antigen of a highly complex pathogen and as a result, after many years of work, has an efficacy (in the prevention of life-threatening episodes) of approx. 30% [5], much less than the 70% efficacy target. This project aims to use a novel computational approach including multiple antigens to design and test vaccines against such pathogens that have been previously difficult to vaccinate against. Success of the designed vaccines within this

project in a pre-clinical model will allow these vaccines to be carried forward externally into clinical testing with the view of reaching the clinic and vaccinating vulnerable individuals.

References

[1] World Health Organisation. (2015). “WHO Health in 2015: From MDGs to SDGs”.

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[3] World Health Organisation. (2021). “Global tuberculosis report 2021”.

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What outputs do you think you will see at the end of this project?

We expect to see multiple patents filed covering different disease areas. Post patent filing, we further expect to publish the data in scientific publications.

Who or what will benefit from these outputs, and how?

The benefit of having patented vaccines is the ability to move these forward as vaccines that have the potential to diminish the global disease burden. These vaccines will be taken forward by larger pharmaceutical companies into clinical trials through partnerships with the aim to "bring to market" effective vaccines where previous vaccine strategies have failed. These outputs will be highly modular; we expect to develop multiple vaccines in parallel to patent with each taking under a year, following which they will be partnered. As a result, this will be an output-heavy project.

How will you look to maximise the outputs of this work?

Pharmaceutical partners have already been identified and/or established to take forward the patented work produced in this project. This aids efficient progression of vaccine development.

Species and numbers of animals expected to be used

- Mice: 2000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice will be used in this project due to a number of factors.

1) There are currently no accepted alternative models for vaccinology studies that are both immunologically and physiologically relevant.

2) Mice are a well-established pre-clinical model within the field, allowing reliable extrapolation of data.

Adult mice will be used due to the need for an "average" immune response, which may be affected by age extremes.

Typically, what will be done to an animal used in your project?

Mice will be vaccinated with a prime and a booster dose(s) of the vaccine to be tested via typically intramuscular injection, subcutaneous injection, intravenous injection, or oral gavage. Adjuvants may be used with intramuscular or subcutaneous routes. Blood sampling via the tail vein will occur at key points during immunisation, including pre-immunisation prime and pre-final immunisation boost. Post the final booster dose, mice will be culled for ex vivo (outside of the animal) analysis. A subset of mice will be killed by exsanguination under terminal anaesthesia when analysis of immune cells in the blood is required. Experiments are expected to last two to three months.

What are the expected impacts and/or adverse effects for the animals during your project?

Impact following vaccination prime or boost:

Mice may experience minimal pain from the administration of vaccine lasting in the realm of a few minutes and/or localised swelling at the site of vaccination that could last for several days. Depending on the vaccination platform, i.e. viral vector (commonly adenovirus) vaccines, piloerection may be expected, which would typically be less than 10 days, but may on occasion be prolonged (in excess of 10 days). Where piloerection is prolonged, mice will be culled if they display additional clinical symptoms.

Vaccine adjuvant would be expected to cause transient (under 24 hours) local inflammation only due to use of the most refined options. In the case of some more potent adjuvants such as incomplete Freund’s (the highly potent complete Freunds will not be used), a granulomatous lump (a small area of inflammation) may occur at the site of immunisation. On such occasions animals will be monitored following immunisation and

any animals showing signs of distress such as prolonged abnormal behaviour or ulceration that breaks the skin will be culled.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Expected severity = moderate. Proportion of animals expected to experience this severity = 10%.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The immune system is highly complex and is yet to be fully understood. As such, a non-animal alternative is inadequate in identifying the efficacy or immunogenicity of a vaccine. However, prior to immunisation in vivo, expression of the vaccine antigen will be assessed in in vitro culture, ensuring only the most appropriate vaccine candidates are taken forward to in vivo experiments.

Which non-animal alternatives did you consider for use in this project?

Use of organ-on-a-chip technologies or organoid systems (artificial organ).

Why were they not suitable?

Neither of these technologies currently adequately reflect the immune response. Indeed, the immune system and thus response is truly systemic; capturing/mimicking the environment of one organ will fail to reflect the true process of an immune response.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This estimation is based on a 5 year project, testing 6 vaccines a year with multiple iterations. Each vaccination will be tested in triplicate and including naive control mice as is expected in the literature.

Due to our experience testing pre-clinical vaccines in a mouse model, we are able to carefully plan experiments, minimising the number of mice required. This is aided by careful consideration of appropriate controls and avoiding unnecessary experimental repetition.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of mice required in each experiment will be determined by careful consideration of the literature, our previous experimental experience and appropriate statistics. Each experiment is planned in triplicate as expected in the literature, however data from the first preliminary experiment will be analysed prior to undertaking the biological repeats to determine whether the full experiment is justifiable.

We have used and will continue to use the NC3R's experimental Design Assistant in our experimental planning.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Data from the first preliminary experiment will be analysed prior to undertaking the biological repeats to determine whether the full experiment is justifiable and for power calculations.

We shall also endeavour to share tissue of naïve control animals where this is possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use a mouse model to test the designed vaccination strategies.

In the immunisation of mice, the most appropriate adjuvant will be carefully chosen in each case for its immunostimulatory action and to minimise the chances of prolonged inflammation or adverse effects in mice. Appropriate choice of adjuvant has the potential to optimise the immune response to immunisation, resulting in a more robust immune response, thus reducing the number of mice required in the study.

Blood sampling will be limited to small amounts and follow NC3R/LASA standards during the studies to prevent hypovolaemia (i.e. low blood volumes) and taken by a minimally invasive method with hygienic materials to minimise chances of infection.

Why can't you use animals that are less sentient?

Less-sentient animals do not have an immune response that is as representative of the human immune response or as well characterised in the literature.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

1) Mice will be group housed where possible to reduce stress and related harm.

2) Environmental enrichment available within the facility will be utilised to reduce stress to the animals.

3) Mice will be monitored regularly post procedure to look for signs of discomfort and distress associated with immunisation at the site of administration as well as behaviour changes.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

LASA Guiding principles on good practice for Animal Welfare and Ethical Review Bodies.

LASA good practice guidelines - Administration of Substances (Rat, Mouse, Guinea Pig, Rabbit). LASA good practice guidelines - Handling and Restraint (Rat, Mouse, Guinea Pig, Rabbit)

Use of the website from the NC3Rs (https://www.nc3rs.org.uk) and LASA (Laboratory Animal Science Association) will also be made.

ARRIVE guidelines (Animal Research: Reporting of in vivo experiments).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We endeavour to stay up to date with the latest literature regarding the 3Rs. We shall work with the Central Biomedical Services and AWERB to ensure that we are aware of the

latest developments and implementations. We shall ensure that PIL holders take up the latest training available.

141. Development and Function of Sensory Organs in the Zebrafish

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

developmental biology, sensory neuroscience, gene function, imaging, drug discovery

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our overall aim is to understand how sensory organs, including the inner ear, lateral line and olfactory system, develop and function in the vertebrate embryo.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Our work contributes to the branches of science known as developmental biology (the study of the changes that occur as a fertilised egg develops into an embryo and finally a mature adult) and sensory neuroscience (the study of our senses, including hearing, balance, sight, touch, smell and taste). We aim to advance scientific knowledge in these areas, including an understanding of gene function, and the regulation of molecules that act as signals within and between cells as sensory organs develop.

We also aim to understand the causes of congenital disorders affecting sensory organs, by using the zebrafish as a model system for the study of human disease and for drug discovery. This can lead to future benefits for human health.

What outputs do you think you will see at the end of this project?

We expect to generate new information, which we will publish as research articles in peer-reviewed open-access journals. The work will generate datasets and scientific resources including imaging datasets, sequence data, assays and protocols, transgenic or mutant lines, and new or repurposed drugs. Where relevant, these will be deposited in public access repositories.

Who or what will benefit from these outputs, and how?

Beneficiaries include our collaborators (including biologists, mathematicians and engineers), developmental biologists, sensory neuroscientists, other zebrafish researchers, and others working in similar fields. Our genetic data will be of interest and benefit to clinicians and patients (e.g. in the development of genetic diagnostic tests). Our outputs will also be of interest to the commercial sector, for example in the development of drug discovery programmes. In the longer term, there may be potential benefits for patients with sensory or other disorders.

How will you look to maximise the outputs of this work?

We will publish our findings in peer-reviewed open-access journals, and will post all manuscripts as preprints on bioRxiv or similar repositories. Much of the work is interdisciplinary, and we work closely with scientific collaborators, maximising the benefit of our scientific findings. We will also disseminate new knowledge through presentation at national and international scientific conferences and other meetings.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 30000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures

Explain why you are using these types of animals and your choice of life stages.

We are using the zebrafish (Danio rerio) as our model system. This species has several major advantages for the work that we do. It is a vertebrate with a high conservation of gene sequence and function to humans. The adults are small, enabling us to perform genetic experiments requiring breeding, and thrive well in captivity. Almost all research is done on the embryos under the age of 5 days post fertilisation. These are optically transparent, allowing us to visualise the development of internal structures using fluorescent markers. Many different genetic and transgenic strains are available to allow us to study gene function and to follow cells labelled with fluorescent markers under the microscope. As the embryos develop in water, they are ideally suited to drug discovery projects, as drugs can be administered to the water in small volumes.

Typically, what will be done to an animal used in your project?

Adult animals will undergo natural matings to generate embryos for experimental work. We typically generate embryos from a group of around 40 individuals, or set up individual pairs of fish for mating. Fish of breeding age may be crossed no more frequently than once every two weeks, and may be bred until the end of their breeding life (approximately 2 years of age).

Each egg lay can generate several hundred embryos. We will examine the embryos up to the age of 5 days post fertilisation using fluorescent microscopy of live embryos, or fix the embryos at different developmental time points to examine patterns of expression of genes or proteins. Live imaging may take place over very rapid time-scales (seconds or minutes), or may involve imaging at intervals over longer times up to 48 hours to generate time-lapse sequences. Imaging experiments have a relatively low throughput (5–10 embryos for each experiment). We aim for imaging experiments to be completed at least in triplicate.

For drug screening projects, initial screens are completed in duplicate, with three biological repeats (embryos) per drug, in 96-well plates. Follow-up experiments may involve testing a drug of interest at different concentrations on larger numbers, for example 50 or 100 embryos, in duplicate or triplicate.

What are the expected impacts and/or adverse effects for the animals during your project?

Genetically altered animals

Most mutations used are fully recessive, and thus adult heterozygous fish are phenotypically wild-type, and display no adverse effects. Some genetic alterations may give rise to a dominant phenotype, such as a change in pigmentation or fin morphology. These effects are not deleterious to health. Fish homozygous for genetic mutations may display a range of phenotypes, depending on the mutation.

Some of these, such as nacre, affect pigmentation, and cause no obvious adverse effects. Others affect the morphology of the inner ear, and may cause balance (hyperactivity,

circling, abnormal posture) or hearing problems. For other mutations, the homozygous mutant phenotype may be lethal at a juvenile stage, or may be unknown. Fish will be regularly monitored as they grow and any showing significant adverse effects of the mutation or other signs of ill health (fungal infections, tumours, emaciation, general listlessness, inability to feed, failure to thrive, abnormal swimming behaviour other than mild balance problems) will be killed by a Schedule 1 method.

Adverse effects from group spawning are not expected. Pair mating is slightly more stressful for the fish and will only be used when necessary; fish will be returned to the family tank as quickly as possible. Fish will be regularly monitored before and after breeding and any showing adverse effects (fungal infections, tumours, emaciation, general listlessness, inability to feed, failure to thrive, abnormal swimming behaviour other than mild balance problems) will be killed by a Schedule 1 method.

Fin biopsy or mucus swabbing for genotyping

Fish generally recover well from fin biopsy (>99% survival rate) and the fin will regenerate within a few weeks to its normal size and shape. Fish will be regularly monitored during and after recovery from the anaesthetic, and during recovery in isolation. Fin clipping is less detrimental to the health of the fish than prolonged periods of isolation. Fungal infection is very rare, but if observed, an anti-fungal agent may be added to the water to aid recovery. Any fish not recovering after one week, or showing other adverse effects (general listlessness, abnormal swimming behaviour) will be killed by a Schedule 1 method.

As an alternative to fin clipping, we will also use mucus swabbing whenever appropriate to obtain material for genotyping. This does not require anaesthesia, and results in less stress and other physiological and behavioural changes than fin clipping, and so is regarded as a refinement of the fin clipping technique (see, for example, Norton et al. (2020), Scientific Reports 10:18212).

Expression of gametes

Fish generally recover well after removal of gametes, and adverse effects are rare. Fish will be regularly monitored during and after recovery from the anaesthetic and any showing adverse effects (fungal infections, general listlessness, abnormal swimming behaviour) will be killed by a Schedule 1 method.

Tracking studies

Fish isolated from the shoal can show signs of increased stress, but no other adverse effects are expected. Fish will be allowed to equilibrate to their surroundings and will be returned to the family tank as soon as the tracking is completed.

Anaesthesia

Where anaesthesia is required, advice will be sought from the NVS and the choice will be consistent with contemporary best practice to be suitable for the nature and duration of the procedure.
Anaesthetic depth will be carefully monitored. Where recovery from anaesthetic is required, recovery will be monitored until the fish are able to swim and feed normally.

Humane end-points

Fish will be regularly monitored during and after recovery from anaesthesia in relevant protocols, such as fin biopsy. Any showing adverse effects (fungal infections, general listlessness, abnormal swimming behaviour) or effects of aging will be killed by a Schedule 1 method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most work will fall in the 'Mild' or 'Sub-threshold' category. Most mutant lines that will be used are recessive embryonic mutations; stocks will be raised as heterozygote carriers, and develop with no obvious phenotypic abnormalities.

Protocols 1 and 3 are classed as ‘Moderate’ to take account of health problems in some of the homozygous fish that are raised to adulthood. A few of our vestibular mutant lines are homozygous viable. From these, we may wish to raise homozygotes for breeding purposes or behavioural analysis. These fish grow and feed normally, and are fertile, but show mild hyperactivity, occasional circling behaviour or temporary loss of balance. We expect this to account for less than 10% of animals raised overall.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals are absolutely necessary for this work, as we wish to understand how sensory organs develop in the context of the rest of the tissues in a live animal. The inner ear is a vertebrate-specific structure, and so specific questions relating to the development of this organ cannot be addressed using an invertebrate model. The zebrafish has the advantage

of lower neurophysiological sensitivity than mammalian models, together with ease of imaging and drug treatments, and is therefore a very suitable model system for the types of studies that we wish to perform.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives for the work that we wish to perform. However, we use in vitro work on cultured cells and computational and mathematical modelling as complements to our work on animal models. For example, we can test the function of molecular variants identified through work on fish in cultured cells, use computational modelling to optimise drug design before testing on fish embryos, or use mathematical modelling to explore the behaviour of genetic networks using data from our experimental work.

Why were they not suitable?

It is not currently possible to recapitulate complex three-dimensional developmental events such as morphogenesis of the semicircular canal system using cultured cells. In the live zebrafish embryo, we can study interactions between multiple different cell types, the dynamics of developmental mechanisms, and the three-dimensional shape changes that contribute to forming the labyrinthine structure of the ear.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Regardless of the number of embryos needed for experimental work, a minimum number of fish need to be kept as live adults for each wild-type, mutant and transgenic line, both to maintain a breeding population and to maintain genetic health and social welfare of the fish. Zebrafish are a shoaling species, and best kept in mixed-sex groups of around 40 fish. Interbreeding from only a few individuals results in poor genetic health. Adult fish are thus maintained at sufficient numbers, and bred at sufficient frequency, to provide a steady supply of embryos for our research purposes. We do not apply statistical tests for these breeding numbers; we maintain an appropriate number of tanks for each line, which is constantly under review for usage levels.

Based on previous usage and our planned work, we need to maintain at any one time: 2 wild-type lines (40 fish per line, per year)

11 genetically altered lines (100 non-ID fish raised to generate 40 ID fish per line)

9 transgenic lines (40 fish per line)

8 transgenic lines crossed to those with genetic alteration (40 fish per line)

We also propose to make at least 5 new lines using CRISPR/Cas9 technology over the course of the project (estimate 500 fish raised to generate 40 fish per line)

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Each lab member will be required to submit a plan of work to NACWO staff before work commences. In line with good laboratory practice, we will write a protocol for each experiment, including: a statement of the objective(s); a description of the experiment, covering such matters as the experimental treatments, details of the experimental material, and the size of the experiment (number of groups, numbers of animals/group); and an outline of the method of analysis of the results. We will follow ARRIVE guidelines for good experimental design including blinding and randomisation, to ensure animal numbers are optimised where possible.

For our breeding stock, the best welfare practice is to keep zebrafish in family groups, as they are a shoaling species. Reducing numbers may not always be the best welfare practice. We will seek advice of the NACWO for keeping minimum numbers of any one strain of zebrafish.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

New techniques such as non-invasive genotyping of embryos (ZEG) are becoming possible. If this proves to be a feasible and robust technique for any of our lines, we will use it routinely to identify carriers of mutations at embryonic stages, preventing the raising of animals with unwanted genotypes and thus minimising animal numbers.

We will make use of efficient breeding or sharing of tissue wherever possible. Some mutant lines have pleiotropic phenotypes and tissues can be shared between projects (e.g. ear and lateral line nerve for the adgrg6 mutant).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use the zebrafish (Danio rerio) as a model system. Almost all experiments will be carried out on embryos under the age of five days post fertilisation.

Fish are maintained in mixed sex tanks of about forty individuals to minimise stress and allow natural behaviours. They breed well under these laboratory conditions and give rise to a large number of progeny.

Embryos will be collected non-invasively through natural pair matings or by allowing fish to spawn in a group over a tank of marbles. Most research work for this project will be carried out on embryos up to the age of 5.2 dpf. If adult fish are isolated from the shoal for pair mating or behavioural testing, they will be returned to the family tank as soon as the experiment is complete.

Where applicable we will use non-recovery anaesthesia for imaging studies.

Why can’t you use animals that are less sentient?

The inner ear is a vertebrate-specific structure, and so specific questions relating to the development of this organ cannot be addressed using an invertebrate model.

Most experimental work will be done on embryos under the age of 5 days post fertilisation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Adult fish are maintained in a state-of-the-art, purpose-built aquarium with constant water circulation and filtration. Fish health and water quality is monitored daily by a dedicated team of technical staff.

The health of adults that are homozygous for any genetic defect will be monitored regularly to check that they are feeding and growing normally.

For behavioural work on adult animals, time spent in isolation will be kept to a minimum, and fish will be returned to a group tank as soon as possible after the experiment. Fish will be monitored regularly after any behavioural testing.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the NC3Rs ARRIVE 2.0 guidelines for experimental planning, conduct and for reporting our experimental findings.

https://arriveguidelines.org/

Zebrafish will be maintained according to current best husbandry practice and guidelines (Aleström et al., 2020).

Aleström, P., D'Angelo, L., Midtlyng, P. J., Schorderet, D. F., Schulte-Merker, S., Sohm, F. and Warner,

S. (2020). Zebrafish: Housing and husbandry recommendations. Lab. Anim. 54, 213–224.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have access to regular information sessions and seminars from the NC3Rs, outlining 3Rs advances and funding opportunities. Information about these sessions is disseminated by email by the Veterinary Services Unit at our establishment to all PPL and PIL holders.

Our Aquarium Team staff also stay up-to-date with advances in 3Rs and communicate these at regular meetings to users of the aquarium facilities and PPL holders.

I will keep the project under review and will implement any new advances (e.g. the ZEG genotyping as mentioned above) if these will bring 3Rs benefit to the project.

142. Organisation of Neuronal Dynamics in Cortical Microcircuits and Related Structures

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Systems neuroscience, Neurophysiology, Cortex, Neuronal networks, Resting state activity

Animal types	Life stages
Mice	juvenile, adult, pregnant, neonate, embryo
Rats	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The cortex is the largest part of the human brain, responsible for our sensorimotor and higher cognitive processes. Using rodents as model organisms, this project aims to advance our understanding of the functional organisation and mechanisms of neuronal networks in this and closely related brain areas.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Understanding how the human brain works is one of the major scientific frontiers of the 21st century.

A progress in this area, beyond the advancement of basic knowledge, holds the promise to revolutionise multiple facets of our life, such as psychology and neurology and related medical care, software, IT and artificial intelligence, human-computer interfaces.

What outputs do you think you will see at the end of this project?

Our research will lead to a better understanding of the way the brain operates in normal conditions, in the presence of degenerative motor disease and under the influence of some classes of psychoactive compounds, such as psychedelics.

Who or what will benefit from these outputs, and how?

The beneficiaries include the neurobiology and neurology communities and our industrial partners.

Amyotrophic lateral sclerosis (ALS) is a disease of motor neurons in the brain and the spinal cord. The loss of these neurons leads to progressive paralysis and most patients die within 2-4 years of disease onset. Presently it has no cure. Studying mice with the same changes to their genes as some human patients is accepted as one of the promising ways to make progress in understanding the disease.

Psychedelic drugs have recently been demonstrated to provide effective treatment for a number of psychiatric disorders, including depression and addiction. However, the mechanisms responsible for this are unknown. Studying this question in mice is a promising way to improve our mechanistic understanding of the way these drugs act, which can in turn lead to improved therapies for humans.

Advances in the two specific directions mentioned above rely on improvements in our understanding of the function and mechanisms in neurotypical human and animal subjects, which is also among the objectives of our work.

How will you look to maximise the outputs of this work?

Collaboration with other academic and non-academic institutions (some already established), publication of research and review papers, publication of results in preprint form, presentation at conferences and workshops, social media posts.

Species and numbers of animals expected to be used

- Mice: 1000
- Rats: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project will use mice and rats. We must use a mammalian species since other animals do not have cortex, the brain structure that is central to our scientific questions.

Mice and rats are widely used model organisms that are easy to bread and maintain in laboratory conditions, which makes them particularly suitable. In particular, mice have become a dominant species in neuroscience research, with particularly strong suite of genetic methods and techniques, some of which will be relied upon in our project.

Most of the animals will have a healthy wild-type or altered genotype, but for the study of motoneuron disease conditions such as ALS we will use mice with genetic modifications that model the disease and result in motor impairments. We however will not let the animals to progress anywhere close to a point at which this could cause severe suffering.

Typically, what will be done to an animal used in your project?

We will use electrophysiological and/or imaging methods to measure the activity of neurons in the cortex and related brain structures of mice or rats. The animals will undergo a general anaesthesia procedure during which a small metal part might be cemented to their skull, and small holes for insertion of electrodes or imaging devices will be made in it. (It is worth noting that similar neurosurgical procedures are carried out in human patients with some major brain disorders). In addition, we might inject into the brain special viruses that modify neuronal activity and/or allow to monitor or influence it by shining light on the neurons. Animals will be given several days to recover before any further procedures.

One type of experiments will be performed in head-fixed animals. Such experiments are particularly suited to mice, and will be carried out only in this species. Mice will be gradually acclimatised to spending up to several hours with their head being held by clamping the metal part (they will be able to move all other parts of their body). This allows to monitor and influence neuronal activity through the previously made holes in the skull. The brain has no pain receptors, thus insertion of tiny electrical probes into the brain does not cause major distress to the animals (and again, similar neurosurgical procedures are done in humans).

A different type of experiments will involve electrodes or optical devices that are small and light enough to be permanently attached to an animal's skull. Such experiments will be carried out in rats and mice. These experiments will allow us to monitor neuronal activity in freely moving animals.

After the initial surgery for implantation/attachment of devices to the skull the animals will be kept for several months. Upon the completion of the programme of measurements the animals will be humanely euthanised. Post mortem, the brain of the animals might be preserved to better determine which neurons were recorded from.

During the training/recording phase of the research, animals will be used to study the effects on neuronal activity of certain classes of psychoactive drugs, or to examine the neuronal activity evoked by non-painful sensory (e.g. visual, auditory, tactile) stimuli or to study partial sensory deprivation (by trimming of their whiskers). As part of this research, animals might have to be trained in a behavioural task. This requires restricting the access of the animals to water, which is used as a positive reinforcer during training.

What are the expected impacts and/or adverse effects for the animals during your project?

Most animals are expected to recover from the main surgery within hours. They could experience pain following it, and for this reason will be given pain killers for several days post op. In a small minority of animals various types of surgical complications, such as intracranial bleeding, might be possible.

Unless these complications are relatively mild, in order to minimise their suffering such animals will be humanely euthanised.

For the study of motoneuron disease conditions such as ALS we will use mice with genetic modifications that model the disease and result in motor impairments. We however will not let the animals to progress anywhere close to a point at which their basic motor abilities, such as the ability to walk, groom or feed, are impaired in a major way.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The project will use mice and rats, the rats will comprise at most 20% of the total number of animals used.

The severity for most animals (both mice and rats) will be moderate. Up to 25% - 30% of the animals (both mice and rats) might be used solely for non-recovery anaesthesia procedures, and some animals (both mice and rats) might be used only for breeding (mild or sub-threshold severity).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The scientific questions that we are interested in can only be answered by using alive and awake mammals, which are the only animals that have cortex.

Which non-animal alternatives did you consider for use in this project?

Computer simulations are the only remotely feasible alternative.

Why were they not suitable?

Without animal experiments the parameters of such models cannot be properly constrained. Furthermore, given our incomplete knowledge of the neural connections in the brain and their functional properties it is likely that such models will not have some key elements of the circuits that we are studying.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on our previous experience and the time it takes to carry out the experimental procedures

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When possible we will be using within-animal controls

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use cutting edge experimental methods which maximise the amount of data obtained from each animal (e.g. the number of neurons whose activity is recorded).

When possible, we will use non-genetically altered littermates to replace wild-type animals from external breeders

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice and rats. These species are widely used model organisms that are easy to bread and maintain in laboratory conditions, which makes them particularly suitable. In particular, mice have become a dominant species in neuroscience research, with particularly strong suite of genetic methods and techniques, some of which will be relied upon in our project.

The electrophysiological and imaging methods that allow observing neuronal activity are uniquely suited for these rodent species. Such methods include high density silicon probes for electrophysiology, 2-photon microscopy, micro-endoscopes and wide-field imaging.

Dozens of transgenic mouse lines targeting specific populations of cortical neurons were developed over the last decade. By relying on this genetic approach, it is possible to express fluorescent proteins (e.g. GFP for synaptic tracing), Calcium-dependent fluorescent proteins (e.g. GCaMP for functional imaging), opsins (e.g. channelrhodopsin, for optogenetic activation or suppression of neurons) and designer receptors exclusively activated by designer drugs (DREADDs, for chemogenetic activation and suppression of activity). The transgenic mouse lines that are likely to be used in the present project include lines that express GCaMP (e.g. Camk2a-tTA;tetO-GCaMP6s line) for imaging and channelrhodopsin for optogenetic stimulation (e.g. Thy1-ChR2-YFP).

Why can’t you use animals that are less sentient?

We must use a mammalian species since other animals do not have cortex, the brain structure that is central to our scientific questions.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

By following the accepted and recommended practices for the key procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3R and publications on experimental methods in systems neuroscience

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Communications from NC3R, NACWOs, following best and new practices in our scientific area.

143. Antimicrobial Resistance and Bacterial Fitness

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Antimicrobial resistance, bacteria, colonisation, chickens

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Evidence suggests that many antibiotic resistant bacteria persist and spread within and between livestock premises despite declining or zero antibiotic use. The overall aims of the work are to acquire quantitative data of how antibiotic resistant bacteria colonise chickens and to investigate the potential for feed and environmental samples contaminated with antibiotic resistant bacteria to act as sources of infection.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Resistance to therapeutic antibiotics is recognised as a growing problem for both human and veterinary medicine, and the need to address the issue is highlighted in the UK 2019-2024 National Action Plan. Little is known about the minimum colonising doses of many pathogenic and most commensal organisms required to establish host colonisation. This is particularly true for antimicrobial resistant (AMR) organisms. This work will fill data gaps that will help in improving our understanding of AMR in the environment and to inform decision makers with regards to best practices for its control.

What outputs do you think you will see at the end of this project?

New information regarding how antibiotic resistant bacteria colonise chickens.

New information on the potential for contaminated feed and environments to act as a source for infection of chickens with antibiotic resistant bacteria.

The findings will form part of a report for the organisation, which will be publicly available following revue.

The work should also result in at least one peer-reviewed paper in a scientific journal.

Who or what will benefit from these outputs, and how?

There are a number of potential benefits from the results of these studies, including: Assessment of the biological relevance of residual resistant bacteria in the environment.

Determination of quantitative dose response data will be valuable for Quantitative Microbial Risk Modelling.

If low levels of resistant bacteria are not transmitted to animals, resources may be saved in relation to high levels of biocide use

Overall, the work will improve our understanding of antibiotic resistance in the environment and help to inform decision makers with regards to best practices for its control.

How will you look to maximise the outputs of this work?

New knowledge will be disseminated in the form of a final report for the organisation, which will be publicly available following revue.

Findings will also be published as at least one peer-reviewed paper in a scientific journal

Species and numbers of animals expected to be used

Domestic fowl (Gallus gallus domesticus): 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The aim of this work is to investigate the colonisation of a living host by AMR bacteria. There are laboratory models that are useful for some research questions, particularly gut models that mimic particular intestinal environments. However, these models do not provide sufficient or appropriate data for the aims of this work. Chickens are an appropriate model for these studies as they are a natural host for the bacteria of interest. Furthermore, chicken is the predominant meat type eaten in the UK and a major source of food-borne bacterial disease, so the properties of AMR bacteria in chickens are of significant interest.

Typically, what will be done to an animal used in your project?

Birds will be infected with non-pathogenic, antibiotic resistant bacteria. Infection will be achieved either by oral inoculation of individual animals, or by natural ingestion following exposure to contaminated feed and materials. Birds will be cloacally swabbed to assess bacteriological status. At the end of each study (typically lasting one week), birds will be killed by a Schedule 1 method

What are the expected impacts and/or adverse effects for the animals during your project?

No adverse effects are expected. Only bacteria that don't cause disease will be used. The two regulated procedures (oral dosing and cloacal swabbing) are both mild and only likely to cause slight short-term stress (for the duration of performing the procedure).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Expected severity for all animals is mild.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of this work is to investigate the colonisation of a living host by bacteria and their are no in vitro methods that model an entire animal.

Which non-animal alternatives did you consider for use in this project?

In vitro gut models and ex vivo intestinal explant models were considered, but neither.

Why were they not suitable?

Both model types can provide relevant information relating to specific aspects of host and pathogen biology, but neither mimic an entire complex animal such as a chicken. In vitro gut models are useful for understanding how bacteria interact with other gut flora in the different sections of the intestine, but don't account for host defence factors, so colonisation in a gut model does not necessarily reflect if/how an organism colonises in vivo. Similarly, explant models only use specific sections of intestine. One of the aims of the planned studies is to generate quantitative data that can be incorporated into mathematical risk models specifically related to chickens. Neither in vitro nor ex vivo would generate sufficient or appropriate data for the aims of the project.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number has been estimated based on the design of the first experiment, achieved following discussion with a statistician, and the number of studies planned.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The advice of one of the establishments qualified statisticians was sought for the experimental design in order to ensure the minimum numbers of birds are used that will provide statistically valid data. Data generated from each experiment will be fed back to the statistician to ensure the experimental design is still the most appropriate.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The first study planned will be used as a pilot. Data generated from this (and subsequent) experiment will be fed back to the statistician to ensure the experimental design is still the most appropriate

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

A chicken model of colonisation. For this project, the chicken is not a surrogate for another host. The methods used to infect (oral gavage or passively by exposure to contaminated feed/materials) and sample (cloacal swabbing) cause minimal or no pain, and there will be no lasting harm to the birds.

Why can’t you use animals that are less sentient?

The aim of the work is to investigate bacterial colonisation of chickens. There are no models using less sentient animals that accurately mimic a chicken.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

There are no foreseen adverse effects arising from these studies. Only mild experimental procedures will be used (oral dosing and cloacal swabbing), and, where possible, these will take place in the morning so that the birds can be monitored in the early stages. Birds will be allowed appropriate environmental enrichments (eg supplementation of standard diet with ‘treats’).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Best practice guidance is obtained from NC3Rs, ARRIVE, IAT and the RSPCA. Publications and articles are also reviewed during the approval process prior to each individual study. Where specialist training is required, inter-institutional exchanges and training visits are organised

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By regularly checking information on the NC3Rs website and regular communication with NVS and NIO who will provide up to date guidance for the duration of the project.

144. Development of Live Bacterial Therapeutics to Treat Diseases with Intestinal Dysbiosis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Microbiome, Immunology, Dysbiosis, Therapy

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify mixtures of beneficial bacteria which have the potential to be used as bacteriotherapies (bacteria used to treat human disease) to treat diseases with intestinal dysbiosis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Imbalances of the intestinal microbiota, termed “dysbiosis” can cause intestinal diseases such as inflammatory bowel disease (IBD) and has further shown a role in the development of other diseases related to diet such as obesity and type 2 diabetes. Ulcerative colitis is the most common type of inflammatory disease of the bowel. It affects about 1 in 400 people in the UK. Crohn's disease affects about 1 in 700 people in the UK.

This work will help us to understand the mechanisms whereby healthy gut bacteria species (microbiome) are able to influence immune responses during intestinal dysbiosis. Specifically, the research will aid in the development of new bacterial therapies to treat diseases associated with imbalances in gut bacteria.

What outputs do you think you will see at the end of this project?

The long-term output of this programme will be to identify the best bacterial therapies for the treatment of IBD, obesity and type II diabetes.

In the shorter-term this will involve making new discoveries on the types and behaviour of common gut bacteria found in people and how these interact with the immune system.

Discoveries made on new bacterial species will be published and available to other researchers

Who or what will benefit from these outputs, and how?

The significant shorter-term output of the programme of work will be to generate data to demonstrate which bacterial cocktails can be used to progress to pre-clinical development in preparation for clinical studies in humans.

In the longer term, we expect our lead novel bacteriotherapies to increase the effectiveness of current therapies for intestinal diseases such as immunosuppressants (drugs that inhibit or prevent activity of the immune system) and immunotherapy (biological therapy for the treatment of disease by activating or suppressing the immune system).

How will you look to maximise the outputs of this work?

The results of the research will be published in scientific journals and presented at scientific conferences. New techniques may be patented and shared with other researchers.

Publication of unsuccessful data or techniques will also be considered where possible.

Species and numbers of animals expected to be used

- Mice: 8000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will use mice to develop colitis or diabetes. Mice are widely used for this type of work to predict the mechanisms of action and the effectiveness to treat disease of new medicines. Importantly, mice are also able to be bred without gut bacteria (germ-free) to enable us to measure the impact of bacterial therapies. All mice used will be adult mice as these will have a fully developed immune system.

Typically, what will be done to an animal used in your project?

The majority of mice on this project will be administered human microbiome or specifically isolated bacteria. Subsequently the gut of these mice will be colonised with these particular bacterial species either using samples obtained from human patients or specific bacteria grown in the lab. Sometimes in order to help these bacteria grow we first need to give the mice antibiotics either in drinking water or by injection into the abdomen to eliminate the bacteria that already live in their digestive tract. At various times they will be treated with our candidate bacterial cocktails given by the mouth which requires putting a small tube into their throat for a very short period of time to deliver the bacteria into the stomach (oral gavage). We will use the least invasive way possible to administer the antibiotics, some of which can be in the food and drinking water but occasionally due to the type of antibiotic we need to do this by injection.

In general the bacterial cocktail will be administered orally under anaesthesia. A subset of these mice will be used in our therapy studies and will be administered with anti-inflammatory drugs by injection to the abdomen. These are treatments that are currently used in hospitals to treat patients. All these procedures will cause minimal suffering to the mice. All the mice will be checked daily by qualified animal care staff.

Mice that have been given bacterial therapies above will then go into a challenge model. These models are DSS colitis which is a chemical given in drinking water that causes an irritation to the gut of the mice. The second model is the development of diabetes where mice will be given high fat diet compared to a normal diet.

When the mice reach the endpoint of the study, defined by a humane endpoint such as weight loss or gain, they will be humanely killed prior to tissue collection. Some mice could have small blood samples collected while they are alive so that we can track the effect of a treatment and a further terminal blood sample under non-recovery anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice typically do not show any altered behaviour when administered bacterial therapies by oral gavage. These bacteria have been extensively characterised within our laboratory assays (analytic procedures in laboratory medicine). Bacterial cocktails are administered orally under anaesthesia to reduce the movement of the animal and ensure all bacteria reaches the stomach. Thus reducing any likelihood of abrasion to the oesophagus and subsequent infection due to the bacteria.

DSS (Dextran sulphate sodium) is a chemical which irritates the lining of the gut leading to inflammation and the development of colitis. For mice receiving DSS to induce colitis this can give rise to clinical signs such as diarrhoea and weight loss. This will be closely monitored (twice a day or more if necessary) and when it exceeds predefined thresholds of either maximal weight loss of 20% or diarrhoea for 48 hours the affected mice will be humanely killed. Mice will be provided with a mashed diet in the bottom of the cage if weight loss over 10% or diarrhoea is observed.

In the model of diabetes, mice will gain weight over time. Mice will be weighed and have a cut off of 40% weight gain or if the blood glucose level is greater than a pre-set value of 25umol. Animals reaching these endpoints will be humanely killed.

Mice will be used to assess the impact of bacterial cocktails on the immune system of healthy mice in the absence of disease. These mice could experience some side effects from the treatments such as lethargy identified by a lack of responsiveness. All mice are carefully monitored and if they reach pre- set observations such as maximal weight loss or display signs of dehydration or changes to normal behaviour they will be humanely killed. We will provide extra bedding and additional support to the mice to minimise these effects.

The type of mice and experimental protocols that we are using are well established, widely used and designed to cause the least pain, suffering and distress to the mice.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mild - 20%
- Moderate - 80%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The immune system is extremely complex. During the development of IBD there is an interaction between bacteria and immune cells within the gut such as white blood cells. These interactions are highly regulated and can not be replicated in experiments carried out in the laboratory.

Which non-animal alternatives did you consider for use in this project?

We are able to grow specific bacteria species from human gut within our laboratory. We then measure the basic interactions of these bacteria with immune cells, such as white blood cells, isolated from human blood samples. These non-animal alternatives allow us to determine the difference between good and bad gut bacteria without the need to use animals. We continuously consider their use and development in light of new experimental data.

Why were they not suitable?

It is not possible to replicate the full complexity of the interactions between bacteria and immune cells using laboratory experiments which do not use animals. Animals with an active immune system remain essential to understand these interactions under conditions within living tissues such as the colon.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on our extensive experience with colitis studies in mice, we use 8-10 mice per treatment group. This is a sufficient sample size to allow for the differences in response we typically observe between individual mice. All experiments with a positive outcome are repeated at least once and a maximum of two times to ensure reproducibility.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We continuously evaluate our methods and experimental results to determine if we can further reduce the number of animals per experiment. We now believe that one of the major sources of variation is caused by the composition of the bacteria between mouse colonies and even between mice in different cages. The effect of mother-offspring proximity during the early stages of life has a strong influence of the composition of an individual's microbiota.

We will employ three measures to address this. First, we will inoculate germ-free animals with defined bacterial types (microbiota) or a purposely designed microbiota guided by our data. The second approach we will employ is to use littermates, who are genetically identical, such that the microbiota is controlled against cage to cage effects. A third approach is using wild type mice with a standard mouse microbiome that are treated to enable colonisation with human microbiome.

We believe these three approaches will lead to less variation between experiments and, as a result, the need for fewer animals to be used.

We also utilise the NC3R's experimental design tool The Experimental Design Assistant (EDA) (https://www.nc3rs.org.uk/experimental-design-assistant-eda) to aid in experimental planning and follow the most recent PREPARE Guidelines: https://norecopa.no/PREPARE to ensure all aspects are conducted to the highest standards. To ensure best practices we follow the updated ARRIVE guidelines [PLoS Biol 2020 18(7): e3000410]

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All work is carried out to laboratory standard operating procedures (SOPs) written by Microbiotica and shared with staff within the facility to ensure standardisation throughout the studies.

Pilot studies will be carried out where any new substance is administered to determine variability in response. These data will aid in optimising the numbers of animals required per group to effectively determine any impact of treatment on the development of colitis or diabetes.

Wherever possible, multiple experiments will be performed on the tissues collected from an individual mouse, including harvesting mouse tissues for experiments or cells that can be frozen and stored. For example, the spleen can be used to isolate mouse immune cells for further assays. Colon can be harvested for analysis of the immune cells within and DNA collected to measure changes in mouse genes with treatment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For this project our idea is that defined bacteriotherapies can prevent the development of or reduce the severity of colitis and diabetes in mice. We used peer reviewed literature to guide our colitis and diabetes model selection alongside in-house data. The chemical-induced colitis model is one we have extensively refined and the addition of the spontaneous colitis model in the IL-10 deficient mice are the most widely used models of colitis.

The chemical induced colitis model has been refined by Microbiotica to administer the lowest concentration of DSS that alters the gut but leads to less weight loss and other harms to the animals. The spontaneous colitis model develops gradually over time and therefore is a less severe model than an induced model.

Why can’t you use animals that are less sentient?

Mice are used in this project as they share many of the same species of gut bacteria as humans. Animals with a more immature life stage do not contain these same bacteria and are not as easily bred and maintained in a germ-free 'bubble' within isolators.

The mice are required to carry out normal habits such as eating and drinking to enable us to measure the impact of orally ingested substances on the gut bacteria.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Germ-free mice are bred and housed in iso-cages within 'bubbles' or large contained isolators which prevent the entry of viruses or bacteria. The cages contain environmental enrichment and mice are typically and where possible housed within social groups of 4/5 animals.

Mice will have daily welfare checks which include observing that animals are healthy and can move freely in every cage, have sufficient food and water and the isolator temperature/humidity readings are appropriate. These details are recorded on observation sheets within a day book for assessment of an individual animal's health status.

We have reduced the maximum age of breeding germ free mice to reduce the risk of twisted guts commonly seen in older germ free mice.

Where novel agents are administered, the duration and frequency of monitoring including periods of continuous monitoring will be used. For example, following administration of novel agents a single animal will be dosed and monitored for one hour. Providing no unexpected adverse effects are observed the other animals on study will be dosed.

For mice which receive oral administration of a mammalian microbiome, this procedure is carried out under anaesthetic to reduce the likelihood of any harm due to movement of the mouse during the process and post procedure checks are carried out. We will continue to work with animal care staff to assess the requirement for anaesthetic use.

Active use of and continuous development of humane endpoints will allow us to continually refine procedures. We aim to test the use of the mouse grimace scale for each protocol (https://nc3rs.org.uk/grimacescales). For colitis models we have an in-house scoring sheet that monitors weight-loss and other signs such as diarrhoea over time.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure best practices we follow the updated ARRIVE guidelines [PLoS Biol 2020 18(7): e3000410]

We also follow the PREPARE guidelines to ensure all best practices are followed by researchers who use animals on this licence. [Lab Anim. 2018 Apr;52(2):135-141. doi: 10.1177/0023677217724823.

Epub 2017 Aug 3.]

In addition, we follow updates to best practice guidelines on The Laboratory Animal Science Association (LASA) and NC3Rs websites.

We will publish to ARRIVE and PREPARE guidelines and where possible data will be presented at scientific conferences.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will use the NC3Rs website to gather up to date information about advances in animal research. This information will be disseminated to anyone involved with this licence including Project Licence Holder (PPLH), Personal Licence Holders (PILs), Named Animal Care and Welfare Officers (NACWOs), Named Information Officer (NIO) and Named Veterinary Surgeon (NVS) and implemented where appropriate.

We have subscribed to ATLA (Alternatives to Laboratory Animals) published by FRAME (Fund for the Replacement of Animals in Medical Experiments), in association with SAGE publishing to keep up to date with recent advances and alternatives in animal research.

145. Fish Homeostasis in a Changing World

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Physiology, Behaviour, Aquaculture, Climate Change, Pollution

Animal types	Life stages
Rainbow Trout (Oncorhynchus mykiss)	juvenile, adult
Salmon (Salmo salar)	juvenile, embryo, adult
Zebra fish (Danio rerio)	embryo, juvenile, adult, neonate
Lumpfish	juvenile, embryo, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to investigate the physiological (functional) and behavioural mechanisms in fish associated with how internal homeostasis (balance) is achieved in response to everyday challenges in nature as well as anthropogenic (human-induced) change, including the challenges presented by differences in water chemistry (exogenous change) and by feeding, exercise and changes during development (i.e. endogenous changes). The specific physiological systems of interest include respiratory gas exchange, salt, water and acid-base regulation, digestion, nutrient absorption, growth and waste excretion, with applications for understanding the effects of climate change and pollution, how to improve the sustainability of aquaculture (fish farming), and how fish can influence global marine carbon and other chemical cycles.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The fundamental physiological and behaviour responses to be studied are important as the “comparative physiology approach”, when applied to fish, enriches our understanding of the processes influencing life on Earth generally, ultimately helping to appreciate the evolution of the vertebrates from invertebrate ancestors, and air-breathing vertebrates (including humans) from our water-breathing ancestors. Furthermore, the fundamental knowledge gained has important applications across several fronts which include:

1) allowing us to predict how aquatic animal populations may be affected by anthropogenic (human- induced) climate change and pollution;

2) finding vital solutions to improve the sustainability of aquatic food production (fish farming or “aquaculture”), and

3) providing important data on how aquatic animals influence ocean chemistry (the opposite of #1, i.e. animals influencing the environment rather than the environment influence animals) that will ultimately feed into global models of atmospheric-ocean movements of CO2 and therefore improve climate change predictions.

It should also be noted that although 19 different fish species are included within this PPL, the actual number of species (and the total number of individual fish) to be used over the 5-year duration of the PPL will be significantly less. For the research that requires wild-caught epipelagic species the project will likely only require experiments on two species that fit this ecological niche criteria. We work with collaborators who help collect these fish from the wild. Based on their advice, the five most likely species to be caught have been listed (mackerel, herring, sardine, horse mackerel and seabass) to ensure the species caught can be worked on under the PPL. Only a maximum of two species will be actively sought at any one collection but the exact species utilised will depend on what is available. The research is possible using any of these 5 epipelagic species. Similarly for the food-based aquaculture objectives of the licence 10 species have been specified (rainbow trout, salmon, lumpfish, sea bass, Ballan wrasse, Senegalese sole, striped catfish, tilapia, barramundi, cod). Research on 3 of these species in the immediate future is ensured by existing funded projects (trout, salmon, lumpfish). However, which of the other species will be used in research over the next 5 years will depend on the needs of our aquaculture industry partners (which can change very quickly) and in turn the success of related funding proposals.

What outputs do you think you will see at the end of this project?

The primary outputs will be new knowledge and publications with relevance to fundamental physiological science, climate change and computer modelling of carbon cycling in the global oceans, and improving the sustainability of aquaculture. In particular we envisage the project contributing scientific communications that include peer-reviewed publications (scientific journals and books), oral presentations at conferences, and outreach events (public talks, school talks, etc.). However, this work will also make important contributions to the aquaculture industry in terms of guidance on optimal conditions for improving the sustainability of food fish production, but also improving the welfare and the 3Rs principles (Replacement, Reduction, and Refinement) for fish used in research (e.g., zebrafish). Such guidance would take the form of communications at aquaculture industry, zebrafish and 3Rs conferences and international networks, aquaculture networking events, magazines, brochures and via social media.

Who or what will benefit from these outputs, and how?

1) Fundamental Physiology - The project will improve our understanding of basic physiology and behaviour of fish and how they integrate to respond to endogenous and exogenous change. Understanding fundamental physiological mechanisms, their integration and control processes is essential to help us determine the adaptations various fish species have to deal with such changes. The fundamental knowledge gained will be communicated (as the data become available) to benefit the comparative animal physiology scientific community via peer-reviewed publications, scientific conferences and seminar presentations.

2) Aquaculture – The aquaculture industry will benefit directly from new knowledge gained concerning optimum conditions for growing fish within intensive farming scenarios, in particular recirculating aquaculture systems (RAS). Each species is likely to have a different optimum level for the water chemistry variables of interest, and these data will be communicated (as the data become available) to the aquaculture industry via a) direct contact with our industry partners (e.g. salmon, trout, lumpfish UK production companies) and aquaculture networks, and b) national initiatives, and c) the aquaculture- facing peer-reviewed literature. For the welfare and 3Rs aspects of model species that are used as surrogates for studying human biology (e.g. zebrafish), the outputs will benefit researchers and research facilities globally by providing novel guidance on optimum conditions for improving the reproducibility of animal research studies. Wider dissemination of these outputs will be assisted by various research funders. Outputs from aquaculture studies can often be directly useful for the industry within the timescale of a 3-4 year PhD study or sometimes earlier.

3) Anthropogenic Change Impacts and Global Marine Carbon and Nutrient Cycles – Outputs in this area will allow predictions and modelling about the potential impacts on whole ecosystems, and the development of management strategies for the conservation of

natural environments, in the interest of fish populations which include fisheries relevant to human food security. Environmental managers and regulatory bodies will benefit from new knowledge regarding the impacts of anthropogenic change (including climate change and aquatic pollution), and importantly leading to improved predictive capabilities. Ecologists, conservation bodies, fisheries managers and global biogeochemical modellers will also be able to make use of the data outputs in protection of aquatic ecosystems worldwide. Outputs that contribute towards modelling of either fish population changes or ocean biogeochemical cycles are likely to require most of the 5 years to produce sufficient information to contribute to such models.

How will you look to maximise the outputs of this work?

The outputs of the work under this license will be maximised by the following approaches:

(1) Scientific Publications. I have a proven track record of over 100 publications, including physiology/behaviour-specific and interdisciplinary papers, invited reviews and book chapters. My BBSRC/NERC funding has enabled most of my recent outputs to be open-access publications and I will seek to continue this approach.

(2) Conferences and seminars. My project team of postgraduate students and postdocs will continue to regularly present their data at international and national scientific conferences. This dissemination route is important for reaching the most relevant research community and establishing new collaborations.

(3) Meeting Organization. I have been the chief organiser of scientific symposia, workshops and conferences (ranging from 100 to 1000 delegates) approximately once every 3 to 4 years during my career, and I will continue with this strategy once COVID-restrictions allow easier international travel. This provides an important platform for my research group’s outputs generally, but vitally widens the networks available for establishing new collaborative partners.

(4) Training and capacity building. Training of early career researchers is vital component of disseminating knowledge and working practices, especially within animal research communities and the 3Rs ethos. I have mentored researchers (MSc and PhD students and postdocs) and hosted collaborative visits for Professors, from USA, Chile, Argentina, Norway, Canada, Germany, and China which has enhanced the career development of those visiting Exeter and helped to spread the novel research findings as well as the techniques and approaches we have developed at Exeter.

(5) Public outreach. My group’s work is regularly the subject of outreach events including talks and debates hosted by various funders, scientific societies, local outreach organisations, and various UK school visits (primary and secondary).

Species and numbers of animals expected to be used

- Rainbow Trout (Oncorhynchus mykiss): 1200

- Salmon (Salmo salar): 1200
- Zebra fish (Danio rerio): 5500
- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All the species to be used are selected based on their relevance either as a model species for the specific physiological/behavioural system to be studied. For example, flounder/killifish are used for their extreme salinity adaptability, or catfish/killifish for their ability to breathe both in air and water. Trout/catfish/barramundi are used for studying digestive physiology of high feeding rate species.

Salmon, trout, lumpfish, catfish, sea bass, wrasse, tilapia, barramundi and Senegalese sole are used for their relevance to the aquaculture industry, and the same species plus sticklebacks, plaice, herring, cod, sardines, and mackerel are used for their relevance to climate change, pollution, or ocean carbon cycle studies. Similarly, the life stage to be used varies in accordance with the nature of the question being asked, and the techniques required for quantifying physiological responses. For example, for developmental impacts of water chemistry clearly early life stages are essential for study (e.g., zebrafish or killifish in the days/weeks immediately after hatching). However, when body fluid samples are required (e.g. blood, urine, gut), then fish of a sufficient size will be used.

Typically, what will be done to an animal used in your project?

A very commonly used experimental protocol would involve single-housing of individual fish for a period ranging from a few days to a few weeks, followed by exposure to a change in water chemistry (within the species' known or estimated physiological range), or feeding a particular size or composition of meal. The physiological responses to such endogenous or exogenous changes would then typically be measured over time scales ranging from minutes to days. For example, an aquaculture fish species would be placed in single-housing tanks (essential to ensure precise knowledge of the amount of food eaten per meal as well as maximising replication and therefore statistical power to determine significant treatment effects within the space limitations of the aquarium facility, and therefore reducing total animal numbers used). The water chemistry would then be altered (e.g. elevation of CO2), followed by feeding on a specific diet at specific intervals (e.g. once per week). Before and at different times after meal ingestion we would then measure variables such as metabolic rate (O2 uptake, CO2 excretion), or ion/acid-base flux (uptake or excretion) via non-invasive analysis of water samples. In some fish body fluid samples may be taken as well (or instead), either via a previously implanted surgical tube in a blood vessel or urinary bladder, or following anaesthesing within their home tanks

and sampling blood via an accessible vein, or urine via the urinary opening. When behavioural responses are also relevant to study, fish movements can be recorded via video cameras placed outside or inside the experimental tank (which can be for solitary fish or in groups). Some additional behavioural tests (such as preference for left or right turns, sensitivity to odours, boldness/anxiety) may require transfer of fish from their isolation or group tank to a separate arena for a period of hours/days where the behavioural test is performed. The most common total duration of an experiment would be 4-6 weeks (with the first few weeks required to acclimatise the fish to a new feeding regime or water chemistry condition), but some climate change or aquaculture studies will require longer (e.g. 12-24 weeks) to mimic the durations most relevant to those settings and maximise the statistical power for detecting subtle changes in growth.

What are the expected impacts and/or adverse effects for the animals during your project?

For the vast majority of experiments we do not expect adverse effects for any life stages to be used. This is because the experimental treatments (water chemistry or feeding regime) involve changes that are well within the normal environmental limits inhabited by each fish species, or aquaculture conditions currently used for growing fish species. A small number of fish will undergo general anaesthesia to have a cannula (tube) surgically placed in a blood vessel or urinary tract. They are expected to recovery quickly and painkillers will be given. In the situations where body fluids are sampled (e.g. blood/urine) without a cannula being previously fitted using the novel technique developed in my lab using an artificial ventilation (gill irrigation) whilst under anaesthesia, there is minimal stress associated with the anaesthesia and sampling process and fish are recovered from anaesthesia with no apparent adverse effects with the potential exception of a minor bruise at the site of the blood sample (as when human’s give blood). The behaviour of the fish is monitored throughout and immediately after sampling and until normal behaviour is resumed.

Fish recover from anaesthesia/cannulation within minutes and would not be used for subsequent sampling involving additional anaesthesia for a minimum of 24 hours.

Single-housing of very social species can create a welfare problem, for example elevation of the stress hormone cortisol. For this reason, for particularly social species (in this PPL it would include zebrafish, mackerel, herring, sardine, and horse mackerel) single-housing will be limited to very short term measurements (e.g. hours to a few days). However, for other less social species, or indeed those that tend to be solitary in nature, there is often no welfare issue with single housing (in this PPL it would include pangasius, flounder, Arabian killifish, tilapia, barramundi, lumpfish, ballan wrasse, Senegalese sole). For some species that form social hierarchies (rainbow trout, salmon, sea bass, stickleback), whilst single housing can temporarily elevate blood stress hormone (cortisol) levels, this impact needs to be weighed up against the normal, low-level occurrence of aggression and associated physical damage to skin and fins that can occur when fish are held in social groups. In our experience with trout, salmon, sea bass and sticklebacks, fish take a few

days to resume normal feeding rates when transferred to individual tanks, but thereafter have a better general body condition and lack of fin damage compared to fish held in groups. We therefore do not foresee welfare concerns (compared to fish held in groups) with the longest period of social isolation that could be used for the longest experiments in terms of single housing (e.g. 24 weeks), or during more typical duration experiments (e.g. 2-6 weeks).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most fish (83 %) will be under protocol 1 that is not expected to result in adverse effects with more than mild severity, with many likely to experience sub-threshold severity.

Protocol 2 (15% of all fish under this PPL) includes the option for a Maximum Burst Swimming Speed test, which is the only step in this Protocol which counts as Moderate severity category. However, <10

% of the fish used in Protocol 2 will undergo this test (i.e. <1.5 % of the total fish under this PPL).

In very few cases (Protocol 3) there is a requirement for multiple steps within a protocol that includes cannulation. This is expected to be < 2% of the total fish within this PPL. These animals have the potential to experience moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are currently no alternatives to using live animals, if we are to gain an understanding the mechanisms involved in the physiological and behavioural responses especially when studying the integrated response of multiple physiological system in fish (e.g., respiratory, osmoregulatory, digestive, sensory physiology). This is true both for the fundamental physiology experiments, and those designed to answer questions concerning sustainability of aquaculture, or predictions about the impacts of climate change or pollution.

Which non-animal alternatives did you consider for use in this project?

(1) There are currently no computational modelling approaches available for studying the integrative physiological and whole organism responses of fish to environmental change.

(2) We are actively engaged in cell culture options when studying a few, selected individual components of a single physiological system. For example, gill and gut cell cultures are being used within our research theme for studying cell types and individual proteins used in gill and gut ion transport processes.

Why were they not suitable?

These gill and gut cell culture systems cannot yet be used to determine the ion regulation responses to in vivo endogenous and exogenous changes (which depends on neural and endocrine inputs among other components), let alone how these may integrate with other physiological systems (e.g. the respiratory or digestive functions of the same organs, the gill and the gut).

However, we are continuing to develop such alternative cell culture models to whole animal experiments that allow us to better predict the mechanisms involved in physiological function of individual organs.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers to be used for each species are based on the predicted maximum number that are likely to be used within each of the relevant projects and their individual experiments that we are currently running and those planned as part of future funding proposals. Whist the experimental design varies between studies, I will use three different typical examples to highlight how numbers are determined.

1) A smaller scale fundamental physiology experiment might require 20 fish in total to investigate how salinity influences the speed of blood acid-base regulation in response to elevated CO2 in a physiologically relevant species (e.g., flounder). This would involve two groups of fish previously acclimated to different salinities (e.g., freshwater and seawater). Once salinity-acclimated, the fish would be fitted with a vascular cannula under anaesthesia, then allowed to recover. Blood samples would then be taken before and at different times (e.g. between 4 and 96 hours) after rapidly elevating the water CO2 level. Based on prior experience of variance in blood gas and acid-base variables in fish, Power

analysis suggests that typically this would require 10 fish per salinity, so 20 fish in total, to have the statistical robustness to determine whether salinity affected the speed of acid-base recovery.

2) A larger scale aquaculture experiment (e.g. using salmon) might involve 320 fish exposed to four different water chemistry treatments (e.g. 4 different levels of CO2) using 4 replicate tanks per treatment (i.e. 16 tanks in total). If running this experiment for 12 weeks we would typically aim to terminally sample a subset of 4 fish per tank every 3 weeks (i.e. 16 fish per treatment sampled at time zero, 3, 6, 9 and 12 weeks). This would require 5 time points x 4 fish per tank x 16 tanks = 320 fish in total.

3) A whole life cycle experiment in zebrafish to determine the optimum water chemistry for maximising reproducibility might require 1,620 fish to be grown from fertilized egg to breeding adult. This number takes into account i) 9 water chemistry treatments (3 CO2 x 3 salt levels), ii) 30 fish per treatment being terminally sampled at 6 intervals during the 6 month experiment to obtain samples for 3 different “tests” (e.g. 10 fish for physiology performance tests, 10 fish for behavioural tests, and 10 fish for measuring growth/developmental markers/gene expression changes). So 30 fish x 6 time points x 9 treatments = 1,620 fish.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Prior to experiments, we use the PREPARE guidelines & checklist and liaise with the animal facility to design the experimental strategy. We also use the NC3R’s research design tool, which feeds into the ARRIVE guidelines. Finally, based on the NC3R’s experimental design assistant (EDA), sample size calculation is used to determine the minimum numbers required to achieve statistical significance, based on our prior knowledge of typical variance for each physiological or behavioural variable of interest within that particular species. Where this involves relatively new species for such experiments (e.g., wrasse which is a newly cultured cleaner fish in the aquaculture industry) we will run pilot experiments to estimate typical variance of physiological and behavioural test endpoints.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Great care is taken to design all the experiments with a view to minimising the use of fish. This includes carrying out pilot studies where relevant, e.g. using zebrafish embryos/larvae before deciding on treatment levels and experimental design for whole life cycle zebrafish studies. The pilot data will then inform estimates of the minimum number required in the larger experiment.

Another example is the fact we always attempt to gain multiple measurements from the same individual fish where this does not unduly impact the severity level experienced by

the animal or the experimental integrity. For example, we might use the same individuals for non-invasive flux measurements, then behavioural tests, followed finally by terminal blood sampling and post-mortem multi-tissue sampling for physiological and molecular endpoints.

A third example is the use of a paired experimental design, e.g. when establishing the changes in blood acid-base chemistry over time (e.g. after feeding or exposure to a water chemistry change) and comparing each time point within the same fish against its own pre-feeding or pre-exposure control timepoint.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

MODEL SPECIES - For much of the research on the physiology and behaviour of fish, model species have been established for which there is a good level of genetic and functional information already available (e.g. zebrafish, trout, salmon, sticklebacks). However, using a much broader range of species is also important to help understand specific fundamental physiological mechanisms. For example, adopting a comparative approach with fish species that have the greatest tolerance to environmental variables such as high salt concentrations (Arabian killifish, flounder), low oxygen (catfish), high CO2 (catfish) etc., provide unique opportunities to learn completely new concepts relating to physiological systems such as salt and water balance, respiratory gas transport and acid-base regulation, respectively. Also, the comparative approach sometimes utilises the natural large differences in species lifestyle, anatomy and physiology to directly compare extremes of function within the fish class (e.g. active, high metabolism active fish like sea bass, salmon or trout v. sedentary, low metabolism flatfish like Senegalese sole, plaice or lumpfish). Secondly, to establish optimal conditions for improving fish culture it is essential to study the species that either dominate use in animal research facilities (i.e. zebrafish), or those being grown for food by the aquaculture industry. Hence this project licence will utilise a number of species relevant to the aquaculture industry (e.g., salmon, trout, lumpfish, wrasse, barramundi, tilapia, catfish, Senegalese sole etc.). Thirdly for studies on climate change, pollution, or the influence of marine fish on global biogeochemical cycles, it is obviously essential to study the species of most relevance to the environmental niches of interest. Sometimes these are based on economic importance (e.g., fisheries such as sea bass, cod, plaice, sole), or based on ecological relevance (e.g., tropical freshwater

zebrafish, temperate freshwater sticklebacks, estuarine flounder, benthic plaice, demersal cod, pelagic mackerel, herring, sardines etc.). This diversity is ultimately essential to provide a better appreciation of the potential for whole ecosystem impacts, and provide appropriate data for accurately modelling and predicting anthropogenic (human-induced) influences.

METHODS - The physiological and behavioural methods used are the only available that can achieve the objectives regarding whole animal responses to endogenous and exogenous changes, both for natural and anthropogenic environmental change, and for conditions within aquaculture. These methods are also the most refined in terms of being able to detect the most subtle changes in physiology (function). For example, the vast majority of our physiological data collected in fish experiments are simple non-invasive measurements of the rate of uptake or excretion of various molecules (e.g., oxygen, carbon dioxide, ammonia, acid, base etc.) simply by taking water samples from the experimental tanks or via sensors in the water (e.g. oxygen). Furthermore, with our current analytical capabilities such measurements are highly sensitive for detecting physiological responses in fish (e.g. 10-fold to 400-fold better sensitivity compared to invasive blood samples for some variables). Such physiological endpoints also have direct implications for understanding fitness of fish in the wild or their performance in aquaculture conditions, and making predictions about how populations will be impacted, or how fish populations will influence global biogeochemistry. Finally, the ranges of treatments to be used (e.g. water chemistry composition) will always be within normal physiological limits (and most commonly within limits currently used within the aquaculture industry). Moreover, physiological and behavioural tests will use the shortest period of time feasible to achieve precise data that can achieve the objectives. Monitoring of fish continuously during relevant individual steps, and at least twice daily during the whole protocol, also ensure that suffering is minimised.

Why can’t you use animals that are less sentient?

Fish are already considered the least sentient of the vertebrates, and this project licence is not about research concerned with using fish to infer more about other vertebrates, including humans. We are studying fish species to learn more about the fish themselves, with a view to better understanding their physiological systems and how they are influenced by change associated with aquaculture, climate change or pollution. For research of this nature that is important for new fundamental knowledge, food security, environmental protection and climate change mitigation, it is therefore essential to utilise animal species that are directly relevant to these topics. Hence studying less sentient invertebrate species cannot provide a suitable surrogate or proxy for fish, and techniques such as cell culture are not capable of determining integrated physiological responses in the whole organism.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The University Animal Facility is well-supported with modern processes and equipment to provide the most appropriate welfare for fish involved in this research. Regulations are in place to ensure fish health and to minimise the risk of infection through environmental transfer.

The vast majority of the projects involve minimal, if any, invasive procedures and therefore pain management is usually not relevant. However, some animals will require tagging or injection of markers under anaesthesia followed by recovery. Current recommendations for brief invasive techniques with mild severity do not include the use of analgesics (painkillers). However, very rarely (<3 % of total numbers) surgery is required to implant a cannula under anaesthesia, followed by recovery and subsequent sampling via the cannula. In circumstances where a cannula is implanted following an incision (e.g. laparotomy to access an intestinal vein) refinement will consist of using a painkiller (e.g. Lidocaine) applied to the wound site to reduce any suffering of the animals post-surgery and anaesthesia. However, the most common cannulae used would be inserted into vessels just below the skin or the urinary bladder via the external urinary opening (papilla) next to the anal fin, or the stomach via the oesophagus. For such cannulae an analgesic would be applied to the skin where cannulae were secured to the body via single sutures (stitches). In the situations where body fluids are sampled (e.g. blood/urine) without a cannula being previously fitted, a novel caudal puncture technique will be adopted using an artificial ventilation (gill irrigation) system that has been developed in my lab. This minimises the stress induced by the anaesthesia and sampling process, and ensures valid blood gas and acid-base analysis which no previous non-cannulation techniques achieved.

At all stages of the project, we will conduct regular welfare assessments to evaluate the impact of procedures and regularly review our experimental processes to minimise adverse effects without impacting scientific outcomes. Any post-surgery animals will be more intensively observed and assessed firstly to ensure full recovery from anaesthesia, and secondly to ensure cannulae remain unblocked and are not causing problems (e.g., with free movement of fish in experimental tanks).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We actively use the NC3Rs Experimental Design Assistant tool to plan our studies, which helps incorporates best practices for the 3Rs into our study design. (https://www.nc3rs.org.uk/experimental- design-assistant-eda)

We will also use the ARRIVE guidelines to inform both the study design and reporting of the result of our work for full transparency and to help others reproduce our findings. (https://arriveguidelines.org/)

The field of anaesthesia and analgesia for fish is fast-developing area of active research, with new interpretations and advice coming out regularly. We will therefore also keep up to date through publications, conference attendance, collaboration and funding body interactions. In particular, we have a number of collaborators that will have the most up to date information regarding appropriate anaesthetic and analgesic regimes for fish.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Since June 2020 the PPL applicant has been an invited member of the NC3Rs Zebrafish Welfare Working Group which provides great opportunities for exposure to, and debate of, new ideas relevant to 3Rs, such as new enrichment and evaluating potential benefits, and useful online e-learning resources. The PPL applicant is also a NC3Rs Project Grant holder and engages with the NC3Rs regularly through the direct reporting on project progress with members of their Head Office in London. He also attends grant holder meetings hosted by the NC3Rs in London, and has attended and presented at 3Rs Symposia, and has had occasional meetings about 3Rs updates and dissemination opportunities with NC3Rs staff. He is also a long-standing member of the Ethics Committee (2005- 2014) and AWERB (2014-present) and so has direct access to updates on new 3Rs approaches, tools and technologies being used at the University and beyond, which are often supplied by a staff member of the NC3Rs. Implementation of 3Rs advances is also facilitated by regular discussions with the Animal Facility Managers, NACWOs and technical support staff, as well as periodic consultations with the Named Veterinary Surgeons.

146. Rho Signalling in Cancer Development, Growth, Detection and Spread

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, signalling, ROCK, metastasis, cytoskeleton

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To further understand cell cytoskeletal signalling pathways crucial for cancer progression and spread, and ultimately put forward new therapies for human use in the clinic.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Metastasis is the spread of cancer cells around the body. Metastatic disease together with therapy resistance are key problems in the clinic for melanoma, breast cancer and pancreatic cancer.

Melanoma and breast cancer patients that develop brain metastases will have much worse prognosis. The usefulness of current cancer therapies is based on the ability to shrink the main tumour mass but we believe it is just as important to stop cancer invasion and spread. Abnormal cancer cell migration is key for metastasis and for that, cancer cells change their cytoskeleton (or their internal skeleton) to spread and survive in distant organs. Key regulators of the cytoskeleton are a family of proteins called Rho-GTPases. Moreover, Rho GTPases also contribute to tumour progression- via their control of tumour initiation , altered metabolism and tumour promoting inflammation.

These signalling cascades are complex and need to be carefully understood. Investigating Rho GTPase protein signalling to clarify their wide ranging involvement in all stages of cancer progression will reveal exciting therapeutic opportunities for cancer patients.

What outputs do you think you will see at the end of this project?

1. Further understanding of cancer progression and spread of particularly aggressive cancers including melanoma, breast cancer and pancreatic cancer. This will be translated into high impact publications and when possible into new biomarker development.

2. Therapeutic use by humans in the clinic: Drugs such as Fasudil and Ripasudil, which target cell cytoskeletal signalling are already making it to the clinic for treating brain spasm and glaucoma in Asia. These classes of drugs would be tested in depth in our pre-clinical mouse studies and future clinical trials.

3. Digital pathology is a speciality of our lab. Carefully preserved and thinly sliced animal tumour tissue can be stained and re-probed multiple times looking for differences in cancer cells that have spread to secondary organs. This data can be shared with researchers physically or as digital tissue slices. This will reduce the number of animal studies required in this Project License.

Who or what will benefit from these outputs, and how?

1. Data generated during this project license will be widely available in open-access, peer-reviewed journals. This has been the case to date for >70 publications of mine.

2. Science communication is a focus of our lab and I have been highly commended as a Brand Ambassador by CRUK; have given over ~150 scientific talks, and have encouraged lab members personally to give ~40 talks themselves. We will communicate our results to the wider scientific community so that findings lead to meaningful therapies making their way into human clinics.

3. We will strive to develop biomarkers of disease progression, which will help direct patients to effective treatment regimes in hospitals.

4. Internal and external researchers may access our bio-banked animal tumour tissue, such as sliced fixed tumour tissue scanned digitally into a PC, or live cells ready to culture in petri dishes outside of research mice.

How will you look to maximise the outputs of this work?

Collaboration where appropriate internally and outside of our University.

Thorough bio-banking of animal tissue to include digital pathology of preserved, sectioned tissue; that is to re-probe and reanalyse tissue for future research interests. Importantly, we will include very early time points in cancer initiation in our studies.

Species and numbers of animals expected to be used

- Mice: 33,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will study aggressive solid cancers only in adult mice because such cancers develop in adulthood.

If we want to study or expand precious human cancer cells, we must use genetically altered mice with impaired immune systems to avoid rejection of the cells.

Genetically altered mice particularly that develop spontaneous tumours are selected based on similarities to human disease, such as symptoms shown during cancer growth and pathology of the tumour.

Mice purchased from reputable suppliers known as wildtype strains are selected to match the background that implantable cancer cells originated in, to avoid rejection of cell growth, and are otherwise healthy and un-altered genetically.

Typically, what will be done to an animal used in your project?

If primary tumour growth is the focus, mice will have tumour cells injected on one occasion typically into the skin on their back intradermally or subcutaneously -or, directly into an organ of origin, such as the pancreas for pancreatic cancer cells. Often the number of cells we inject has been chosen through our own previous experience, or, has widely

been cited in the literature by researchers in mice. Guided ultrasound injection will always be favoured to open surgery to improve welfare and recovery time of animals.

Importantly, primary tumours must not exceed 1.5 cm3 in volume, or this volume collectively for two individual tumours growing. Any other unwanted tumour or growth such as a papilloma must not exceed 1 cm3 in volume. Any growth or tumour interfering with normal behaviours in mice such as eating, drinking or movement may result in termination of the mouse prior to maximum volumes being approached.

A proportion of animals with untreated tumours will be culled at early time points (7-20 days), where early interaction of cancer cells with surrounding tissue and organelles such as white blood cells can be explored.

Specifically to promote metastasis:

(1) Lung metastases: mice are placed in a heated airflow chamber to encourage vasodilation of the tail veins, then transferred to a restrainer to receive a single intravenous injection of cancer cells.

(2) Liver metastases: Cancer cells are injected once into the spleen directly via ultrasound guided injection under anaesthesia, or through an <0.5 cm opening into the body cavity directly near the spleen that is sutured closed. Mice return for a second surgery for removal of the spleen 7-10 d later. Removal of the primary tumour in the spleen gives more time for secondary lesions to appear in the liver.

(3) Brain metastases: will require a single intracardiac injection, which would be performed using guided ultrasound under anaesthesia.

A proportion of animals with untreated tumours will be killed at early timepoints (7-20 days), where micrometastases may have formed. Pathological sections of these early lesions may reveal trends in cancer spread as well as changes in the surrounding tumour microenvironment.

External tumour monitoring- Tumours are calipered manually regularly in order to obtain tumour growth curves for all cells we inject via different routes. No single primary tumour must exceed 1.5 cm3 in volume, or this volume collectively for two individual tumours growing. We also use tumour volume data to enrol mice in what's called 'rolling recruitment' onto therapy dosing studies. For example, to inject mice with a control substance versus active drug solution 5 days a week when tumours reach between 50-80 mm3.

Internal tumour monitoring- Can be non-invasively imaged using anaesthesia typically under 30 minutes using MRI scanning per animal, 1-2x a week. No single primary tumour must exceed 1.5 cm3 in volume, or this volume collectively for two individual tumours growing.

Drug treatment regimes- Not all tumour tissue generated in mice requires drug treatment because often we want to look at tumour tissue or digested tumour cells ex-vivo, or at pathology to analyse trends in the tumour microenvironment. Typically treatments we introduce into mice are safe and have been administered widely in the literature. Animals are dosed 5-7 days a week, once a day via either oral gavage or intraperitoneal injection. An animal can only be dosed twice in any 24h period, which could be two intraperitoneal injections, for example. Animals can expect to be treated for 2-4 weeks.

We strive to use effective dosing regimes which avoid daily dosing if that improves welfare for animals, yet effectiveness of our compounds thus far have been optimal with daily dosing.

What are the expected impacts and/or adverse effects for the animals during your project?

Breeding Protocols (1 and 2)

Offspring bred through **mild severity breeding Protocol 1** should not be capable of spontaneously forming tumours or experiencing more than mild, transient harm from a small ear clipping taken for genotyping purposes and doubling up as a means of animal identification. However, if any tumour or growth is confirmed, animals shall be assigned to **spontaneous tumour development Protocol 3**. Prior to weaning, any mouse correctly genotyped as capable of tumour forming will be transferred to m**oderate severity breeding Protocol 2** before the visible tumour has appeared, and before clinical signs and symptoms of disease are expected. Such mice are capable of forming tumours spontaneously in expected organs and within expected time frames. Mice are transferred to **moderate severity spontaneous tumour development Protocol 3** for potential tumour monitoring and disease progression to occur if:

(i) Tumour(s) are identified, which might have been through palpation or non-invasive imaging techniques. Importantly, we will cease to use breeding stock animals where tumours have been identified to remove the reproductive burden from the animal.

(ii) We wish to chemically activate a gene expressed by correctly genotyped offspring, which will lead to tumour formation and the possibility of moderate severity. For example, antibiotics given in the drinking water or a course of intraperitoneal injections to initiate tumour development.

Tumour bearing mice

Progressive disease will be experienced by mice with up to moderate severity with time as primary and in some cases secondary tumours develop in moderate Protocols 3-5 of this license. Mice can experience weight loss (but one should also consider the overall body condition of the mouse as signs of tumour take over). Expected tumours will be allowed to progress to the size of the research question at hand, which may not by default need to

approach project license volume limit of 1.5 cm3. The exception is that additional, off-target tumours or growths such as papillomas must not exceed 1 cm3.

Our staff have detailed Welfare Guidelines on health monitoring in tumour bearing mice, and which combination of signs and symptoms must result in humane culling of the animal deemed to be suffering lasting moderate severity. No tumour should interfere with feeding, drinking, defecation, urination or movement and overall curious and alert nature of mice.

Researchers performing procedures and administering treatments

Small animal procedures carried out by competent staff including injections, gavage or inhalation anaesthesia should equate to mild, transient discomfort for mice. Particularly with daily drug dosing, these procedures will be done at the same time each day and refrain from disturbing natural light and dark cycles in the mice.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of animals used in our license will experience moderate levels of harm, mainly due to tumour growth at a primary or metastatic location in the body in **Protocol 3-5** in this license. All immunocompromised mice also have the ability to experience up to moderate levels of harm because of their risk of infection.

Stock breeding mice in **Protocol 1** are expected to experience only mild severity due to a small ear clipping being taken for genotyping and their simple DNA alterations lacking the capability to form tumours alone. Early DNA testing will confirm if offspring generated in **mild severity Protocol 1** are able to develop tumours naturally, and mice will be moved as early as possible to **moderate breeding protocol 2** as a precaution. When a tumour is identified in any animal on protocol 2, or, we actively administer chemicals to animals to induce tumour formation: individuals will be transferred to moderate spontaneous **tumour development Protocol 3** and the disease permitted to progress.

Animals in **Protocol 4**, where tumours are surgically implanted superficially in layers of the skin or into internal organs under anaesthesia- will all experience no more than moderate severity. Where possible and to lower harms to the animals, we will use guided ultrasound injection of cancer cells into the body cavity rather than open surgery. Moderate harm is likely reached by most mice in this protocol because we often treat with daily doses of therapeutic compounds post- implantation of cancer.

Animals entering metastasis **Protocol 5** are all expected to experience moderate harms because the models promote the spread of cancer cells to multiple organs including the liver, lungs and potentially bones.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal models are essential for studying complex diseases such as cancer; and a requirement for testing pre-clinical and promising scientific therapies that we would hope to make available to patients. Mice have a short life span, fast reproductive cycle, known genetic background, close genetic and physiological similarities to humans, and recapitulate many aspects of the genesis, progression and clinical course of human cancers.

Which non-animal alternatives did you consider for use in this project?

Human samples

Using patient skin tumours, we have found the top signalling pathways (ROCK1/2, NfKb, JACK/Stat) upregulated by tumours that resist and grow despite being on anti-cancer treatments, and consequences of this including converting macrophages to tumour-promoting, and an addiction to ROCK signalling and subsequent vulnerability to ROCK inhibitor treatment.

We use fresh human blood samples to study the crosstalk between cancer cells and monocyte-derived macrophages.

Bioinformatics and pre-existing data mining

We consult Databases such as The Cancer Genome Atlas for unbiased information comparing gene expression of metastatic versus primary tumour cancer cells from patients. This helped focus our attention on the ROCK/ cytoskeletal signalling cascades.

In vitro and ex-vivo cell culture

Our in vitro work aims to deconstruct complex events between cancer cells and the surrounding tumour microenvironment; which is complemented always by in vivo work to get a holistic understanding of each component of surrounding tumour tissue. For example:

(A) Co-cultures: we plate co-cultures of cancer cells to look at the interaction with either monocytes and macrophages, endothelial cells, or fibroblasts. We can also culture cancer cells with just a sample of solution called conditioned media, which specific

immune cells cells have been growing in: to see whether secretions in the solution from immune cells can influence cancer cells.

(B) Transwell assays are used in our lab to study cancer cell migration in response to chemical chemoattractant(s). We are able to better understand how chemoattractant gradients promote cancer cell invasion and support potential movement across barriers, such as the endothelium, to metastasize. We have shortlisted promising chemoattractant candidates that may promote migration and invasion in future in vivo assays.

(C) Organ on a chip technology is something we are applying to metastasis and optimising in the field of pancreatic cancer in collaboration with a leading manufacturer of the chips. Pancreatic cancer cells are injected and flow across a chip in a stream- and we assess their ability to attach to a layer of endothelial cells; migrate through it and to establish micro metastases in a layer of liver cells. Overall, this is another tool to mimic in vitro the metastasis process, from the circulation of cancer cells in the bloodstream to the formation of liver metastasis.

(D) Collagen I gels mimic the extracellular matrix that cells encounter in vivo. Cells are seeded on top or embedded within the gels, depending on the final goal of the assay (e.g. video-lapse microscopy to study cell morphology and migration; extraction of protein or RNA to study cell signalling).

(E) Collagen microtrack work provides an ideal engineered model of cell migration to investigate how tumour migrating cells with increasing metastatic potential respond to different stiffness and degree of confinement. Polydimethylsiloxane (PDMS) stamps are designed with different track-shapes and restriction points, which recapitulate human tissues and tumours. We fill the unique patterned stamps with collagen solution, which sets. Cells of different metastatic potential are then added and finally migration is monitored over time using specialised microscopy techniques.

(F) Invasive growth assays involve culturing cells as spheres (mini tumours) embedded into a collagen gel and measuring both tumour growth and individual or collective invasion into the collagen gels.

Why were they not suitable?

In vitro cell assays we use contain a simplified mixture of deconstructed tissue that lacks structural complexity and many molecules that would be found in the tumour microenvironment in the skin or organ of implant in a mouse. Whatever our intervention on a growing tumour, the outcome will have the chance to include interactions of surrounding molecules and structures at the site of implant, as well as in the whole body of a mouse.

Liquid cultures could be flowed through organs on chips for example, but still lack the complexity and array of biological molecules that could be influencing drug treatment, the ability to grow and metastasise.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers in experimental groups are estimates based on the size of the effect that we expect to see between our various test conditions, standard agents and controls (using our own previous data, pilot data and/or that seen in the literature) and individual variability within groups, and will be different for each experiment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used NC3Rs' experimental design assistant which helps with the experimental design and statistical analysis plans by providing support for randomisation, performs sample size calculations and advises on the most appropriate statistical analysis methods for each set of experiments.

Whenever possible use of in vitro assays will reduce the numbers of animal required, having the possibility to perform valuable preliminary screening in vitro in 2D as well as 3D collagen scaffolds.

Ultimately, potential therapeutic targets we will identify during our studies will needed to be tested in vivo. However, the minimum number of animals will be utilised for each experiment performed under this programme of work while maintaining a reliable and measurable output.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use pilot studies for new or what could be very large initial experiments. Documented findings both positive and negative will be recorded in order to implement into future work.

Our lab specialises in digital pathology, and we will offer and make use of tissue sharing; especially as we have been able to re-stain the same mouse tissue up to 7 separate times to date. We will ensure tissue we require does not exist from collaborators, before running new in vivo experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

To intervene with cytoskeletal cell signalling events during cancer or administer novel therapeutics, we rely on genetically altered mice which spontaneously develop cancer; implanting tumour tissue into healthy mice or inducing tumour development in genetically able mice with chemicals.

Spontaneous tumour development models

Generically altered animals purchased or bred in-house which develop a primary tumour within a typical 2-6 month window are the KPC pancreatic cancer strain. Regular palpation of the primary tumour, if not internal MRI or ultrasound imaging will ensure primary tumours are detected and monitored to take the minimum tissue required to answer the study at hand, whilst balancing the welfare of the mice and ensure metastases are only generated in Protocol 5 animals. For example, sufficient tissue taken earlier in life may prevent GA mice prone to develop pancreatic tumours from suffering from abdominal swelling similar to a pregnant mouse as the tumour grows.

Tumour implantation (subcutaneous and orthotopic)

The majority of implanted tumours are within wild-type commercially available C57B/6 mice. Implants are located subcutaneously in a single flank and cause no hindrance of gait or natural behaviour in study animals and tend to remain compact and without skin lesions during a study. Orthotopic cancer implants are into the organ of cancer cell origin. These operations are done aseptically and under general anaesthesia, with pre and post-operative pain relief given as necessary. The lowest volume of cells or pieces of tumour tissue are used, and longer term studies mice will be monitored with ultrasound or MRI imaging briefly under anaesthetic, to follow the nature of cancer growth. As tumours develop, these mice are typically assigned to treatment 2-5 days a week via oral gavage or IP injection.

Models of metastasis

Cancer cells are typically injected into the tail vein- but in certain cases directly into the spleen or via guided intracardiac injection into the left heart ventricle under recovery anaesthesia. We want to study micro-metastases at early time points (<21 days) in a proportion of animals. It is highly likely that we avoid subjecting these mice to metastatic

growth associated disease in this time frame such as lesions found in the bones; but mice will be closely monitored daily and they will be culled if they present adverse clinical signs due to metastatic tumour growth such as persistent inappetance, hunched posture, facial grimace, loss of 15% of body weight or prolonged diarrhoea or neurological/ behavioural changes such as circling behaviour or heat tilt which may indicate brain lesions advancing. A single event such as a seizure that could happen as brain metastases advance, or breathing difficulties such as lung wheezing or rattling as lung metastases form are also humane endpoints.

Immunocompromised xenograft models

With human patient cancer cells available, we will use immunocompromised (disabled immune system) mouse xenograft models. These animals will be maintained in a barrier system, handled in airflow hoods and housed in individually ventilated cages to limit pathogen exposure and unnecessary disease.

Why can’t you use animals that are less sentient?

We need an adult, living system where the dynamics of cancer progression can be altered using cytoskeletal therapies. Less sentient animals often diverge from their similarity to humans genetically, and lifespan may not allow for longer term treatment regimes.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Indeed we have made a refinement in asking for ultrasound guided implantation of cancer cells rather than open surgery here in our PPL application.

Where possible, study animals will be handled by the same researcher at a similar time each day to provide routine and mice to become familiar with handlers and their scent.

We will document the growth curves of tumours from old and new cell types we implant, based on the cell number implanted and route of administration. As we then identify and put forward new cancer therapies, we can select the most appropriate animal model set up for purpose.

Cancer cells are fluorescently labelled where possible to enable their tracking in vivo, as well as in pathology sections at histology. We will consult the literature when necessary to ensure that chosen tags remain non-immunogenic.

Embark on digital methods of external tumour calipering in place of subjective manual calipering if available.

Every study we complete has full notes in a debrief, where refinements will always be sought.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All researchers using this licence will read and follow advice in two key papers published discussing research animal use in cancer:

(1) Guidelines for the welfare and use of animals in cancer research by Workmann and Colleagues in 2010.

(2) Guidelines for planning and conducting high-quality research and testing on animals by Adrian Smith in 2020.

(3) PREPARE: guidelines for planning animal research and testing published by Adrian Smith two years earlier, outlining a quality control checklist for all stages of study using animals.

Users of this license will also visit the NC3R's online where excellent information is provided regarding “The Responsibility in the use of animals in bioscience research”, as it sets out the expectations of the funding bodies for the use of animals in research

Without question we will remain up to date in how best to conduct experiments using mice from emerging literature, workshops or legislation.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

PIL holders working under this licence will regularly attend NC3R, BPS or similar meetings to keep abreast of new developments, and to maintain awareness of current best practices.

147. Optimising Lung Therapy using Large Animal Models

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Lung disease, Gene Therapy, Lung, Sheep, Pig

Animal types	Life stages
Sheep	adult
Pigs	juvenile, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to evaluate the safety and efficacy of novel gene delivery approaches for lung disease. The use of large animals provides a system with direct clinical relevance and will facilitate translation to human studies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Lung disease is one of the three biggest killer disease areas in the UK, alongside heart disease and non-lung cancers. It kills 115,000 people each year, the equivalent of one person every five minutes and it places a huge burden on our health care services, accounting for over 700,000 hospital admissions and over 6.1 million hospital bed days in the UK each year.

Research is urgently needed to improve the way we prevent, treat and care for people with lung disease, from the earliest to the end stages of life.

Ordinarily new treatments are developed through a process that involves early testing in preclinical models – and in most instances these models involve the use of mice. Unfortunately this system is largely failing to deliver the new drugs necessary to keep pace with the upward trends in lung disease. New models are urgently required that improve on existing systems.

What outputs do you think you will see at the end of this project?

Of all the potential outputs from this work best case scenario would be the development of new treatments for patients with lung disease that obtain approval for clinical use and have the potential to increase life expectancy and/or quality of life. Other expected outputs would be research publications, conference abstracts and seminar presentations that would inform the research community and stimulate further development. Public/Patient involvement and engagement activities to improve the understanding of such gene-based treatments will also be an important output from these studies. In addition, the early capture of intellectual property arising out of such novel research could assist in securing a competitive advantage for research and commercial activity arising out of the UK, and attract research and development investment from global business as has been demonstrated in our Cystic Fibrosis gene therapy program

Who or what will benefit from these outputs, and how?

In the context of patients, this project will potentially provide a new category of treatments for serious lung diseases and provide the healthcare professionals responsible for their care with more effective options. Ultimately, that benefit will likely be realised in the form of improved quality of life, and increased life-expectancy. Our Cystic Fibrosis gene therapy program, was recently licensed by a large pharmaceutical company and is rapidly progressing towards clinical trials in patients. Our studies will also assist in the design of such clinical trials.

How will you look to maximise the outputs of this work?

We operate as part of a UK wide consortium aimed at developing gene therapy for lung disease and this maximises collaboration between researchers with a broad range of relevant expertise. An important output will be research publications, conference abstracts

and seminar presentations that would inform the research community and stimulate further development. Public/Patient engagement activities to improve the understanding of such gene-based treatments will be another important output from these studies. In addition, the early capture of intellectual property arising out of such novel research could assist in securing a competitive advantage for research and commercial activity arising out of the UK, and attract research and development investment from global business as has been demonstrated in our Cystic Fibrosis gene therapy program which was recently licensed by a large pharmaceutical company.

Species and numbers of animals expected to be used

- Sheep: 60
- Pigs: 60

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Sheep and pigs have been used as models for many years to predict the safety and efficacy of drugs for lung disease. The benefits of studies in large animals were realised in translation from our studies in adult sheep into a large clinical trial involving people with cystic fibrosis in the UK, which demonstrated that repeated doses of gene therapy had a meaningful effect on the disease, slowing its progression. The use of sheep was prompted by the realisation that mice were not predictive of humans in this context and a larger species with more relevant physiology and anatomy was required to model the delivery of new treatments to the lung and the assessment of benefits due to those treatments. The use of the sheep can be extended to support the translation of and products for a range of lung disease in adults such as alpha-1-antitrypsin deficiency, interstitial pulmonary fibrosis, pulmonary alveolar proteinosis and even some respiratory viral infections. Similarly, the use of pigs was prompted by their size and immunological similarity to humans making them a far more suitable model for specific inflammatory syndromes such as Acute Respiratory Distress Syndrome (ARDS) and Sepsis, where animal models are generally lacking.

We believe that the extension of our translational models to include piglets will help to tackle neonatal and juvenile human diseases such as surfactant deficiencies, rare but lethal conditions such as surfactant B deficiency which currently has no effective treatment and children born with these disease typically die within a matter of months. We believe that our gene therapy platform could be an effective option. Again, the larger and more physiological relevant neonatal piglet animal model will allow for therapeutic efficacy development and may overcome the limitations of cell line and rodent models.

Typically, what will be done to an animal used in your project?

Typically the animals will be anaesthetised to allow the therapy to be administered either directly into the lungs though an endotracheal tube or by inhalation. They will thereafter recover from anaesthesia and will be monitored for any adverse responses to the treatment. Where necessary we are able to limit the proportion of the lung that receives the treatment with direct application to individual lung segments. Once recovered the animals will be kept for a number of days to allow expression of the delivered gene. In some cases gene expression may be assessed by anaesthetizing animals periodically and sampling tissues and/or fluids from the areas under study. The animals will be killed at the end of these experiments in order to allow us to examine the lungs at post mortem and take samples that would otherwise be unavailable

What are the expected impacts and/or adverse effects for the animals during your project?

The lung has tremendous reserve capacity, therefore, it is our experience that the animals do not suffer from adverse effect in the form of shortness of breath, coughing, or difficulty in breathing, hence appearing essentially unaffected when compared to control animals. We do know however from previous studies that there can be a mild immune response following delivery to the lung which is short lived and resolves without any intervention and is not accompanied by any adverse clinical effect.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The fact that we have to anaesthetise animals to perform bronchoscopy or related procedures, renders most of the procedures outlined in this license moderate severity. However, it is our experience that sheep and pigs tolerate anaesthesia and delivery very well and recover to standing within 10-15 minutes after cessation of anaesthesia, and subsequently experience no apparent adverse effect.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Lung tissue comprises a number of different structures and types of cells. In addition, the way in which the lung responds to treatment interventions is complex and involves the interplay of multiple systems and factors including the circulation, and the nervous and immune systems. Whilst we can investigate aspects such as which cell types are targeted by our gene therapy in cells in culture to a limited extent, it is currently not possible to use these systems in isolation to predict how the whole lung will respond, particularly from a safety perspective.

Which non-animal alternatives did you consider for use in this project?

Where possible we do use alternatives to live animals in our studies. For instance, in relation to gene therapy we employ human, mouse and sheep cell culture systems to screen for efficacy in gene transfection. We also use cultured precision cut lung slices (PCLS) from sheep, pig and human lung to assess the efficacy of our treatment. Such studies can prove valuable in screening potential gene transfer agents prior to assessment in mouse, and then in larger animals.

Why were they not suitable?

In vivo studies are essential, because only in living animals can the influence of an intact immune system be properly assessed – an important aspect of gene therapy using viral vectors. Studies on the whole lung of the larger animals also allow us to establish clinically relevant delivery methods, sampling and dose-finding studies

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers have been estimated based on the numbers used over the course of the previous license and more than 20 years expertise. We can generate useful data from small n=3 or 4 per group studies since there is such a clear & measurable distinction between control untreated animals and animals that receive the gene therapy treatment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Strategies we have and will continue to use where practical include longitudinal sampling studies using bronchoscopy to collect duration information on the same animal at multiple time points, something that is not possible in rodent studies. We also typically deliver the gene therapy product to one lung of the animal and use the other side as a "within-animal"

control tissue to avoid using separate animals as control. We also consider animal numbers during the submission of the Study Request Forms to our named vets for approval.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We regularly perform pilot studies to validate protocols on ex vivo lungs prior to small pilot studies on live animals to make sure that any new delivery protocol is well tolerated. The majority of our studies are performed in a staggered manner and will wait to see the outcome of individual animals before completing the rest of a group with the same protocol. If we have spare control tissue we often use it to prepare and freeze PCLS that we can use to pilot subsequent studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use adult sheep and neonatal/juvenile piglets (from 4 weeks up to 2 years) to model the delivery of gene therapy treatments to adult and neonatal lung and generate data on the safety and efficacy of the treatments. All delivery and sampling protocols on live animals are performed under general anaesthesia to minimise suffering and distress. In previous studies with our lentiviral-based gene therapy treatments we have not observed any lasting harm from our protocols.

Why can’t you use animals that are less sentient?

We are using adult sheep and juvenile pigs as we are trying to understand the impact of therapy intended to be delivered to an adult human lung. Similarly with the piglet studies we are developing a gene therapy that is intended to be administered to neonatal infants with a lethal genetic condition. The respective lung size, physiology and immunology of the chosen species allow use to obtain valuable information on the safety and efficacy of treatments and also give valuable information on dose-finding and longitudinal sampling protocols that can be translated to the clinical situation. This would not be possible on less sentient animals and we believe we have chosen the most suitable animals for our studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will utilise the considerable expertise of our local veterinary anaesthetists and animal technicians within our facility to refine our protocols and how the animals are routinely monitored based on developments in the field.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will make use of the resources at the NC3Rs website https://nc3rs.org.uk/who-we-are/3rs and the NC3Rs regular newsletters to keep up with advancing knowledge and technology

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am a member of our local AWERB committee and so participate in reviewing licence applications and amendments which gives exposure to changing practices. Similarly attending conferences and reading relevant literature increases awareness of advances that allow for refinement of protocols. The regular local review of study request forms by our named vets and the in-house interaction with our veterinary anaesthetists and animal technicians is also valuable in continually refining our protocols.

148. Elucidating the Mechanisms of Plasma Cell and Memory B-Cell Differentiation in the Germinal Centre Reaction

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Immune system, Vaccination, Infectious disease, Autoimmune disease, Blood cancer

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To better understand how B lymphocytes differentiate into high-affinity plasma cells (PCs) and memory B cells during the germinal centre (GC) B-cell reaction of the T cell-dependent immune response in order to develop more effective strategies for vaccination and the targeted inhibition of activated B cells in autoimmune disease and malignant B cells in lymphoid cancers.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

B lymphocytes generated in T cell-dependent immune responses are critically involved in several diseases, either through mediating protective functions in infectious disease, through their aberrant activation in autoimmune disease or through oncogenic transformation in blood cancer.

First, the generation of high-affinity antibodies in response to microbial infection and vaccination is fundamental for the organism's immunological protection against infectious agents. Central to this process is the germinal centre (GC) B-cell reaction of the T cell-dependent immune response. The GC reaction produces long-lived plasma cells (PCs) that secrete highly specific antibodies against the immunizing pathogen, often establishing life-long immunity.1-5 However, not all pathogens or vaccines induce durable antibody responses, leaving the body susceptible to subsequent infections, a long- known circumstance and more recently highlighted by the SARS-CoV2 pandemic.6-8 Thus, there is a need to better understand the molecular mechanisms underlying PC differentiation in the GC, because this may inform the development of novel strategies to enhance the humoral immune response to vaccination and increase durability.6

Second, several autoimmune diseases are associated with GC-derived PCs that secrete self-reactive antibodies. Thus, insights into how PC development occurs during the GC reaction may provide the mechanistic rationale to inform new treatments that inhibit biological programs activated in pathogenic GC-derived PCs of inflammatory autoimmune conditions such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA).9-11

Third, the vast majority of B-cell lymphomas and PC malignancies originate from the malignant transformation of GC B cells or B cells that have undergone the GC reaction. Indeed, cancers of B cells represent a major public health concern in the UK. In 2018, 14,000 new lymphoma cases and an additional 6,000 myeloma cases were diagnosed, with more than 4,900 and 3,100 reported deaths, respectively (numbers from Cancer Research UK). Understanding the molecular mechanisms of GC B-cell differentiation is a prerequisite to better understand the transforming events leading to lymphomas and PC malignancies, which will provide the basis for developing more specific and effective anti-cancer drugs.12

Thus, the new insights into how B cells differentiate into high-affinity PCs and memory B cells upon antigen-activation arising from our proposed work is expected to have a significant impact on either the prevention (infectious disease) or targeted inhibition

(autoimmunity, blood cancer) of several diseases. Our goal is to exploit the findings made in mice for rapid translation into public health (vaccination) and the clinics (treatments of autoimmunity and blood cancer).

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What outputs do you think you will see at the end of this project?

Our proposed studies are expected to identify previously unknown genes or pathways that 1) provide candidates for a targeted modulation of plasma cell (PC)-precursor development in the germinal centre (GC) that may yield PCs with longer durability or in greater numbers, thus enhancing the efficacy of the antibody response to vaccines directed at preventing infectious agents, or 2) represent Achilles' heels that are suitable targets for a drug-mediated inhibition of pathogenic plasmablasts secreting autoantibodies as well as aggressive GC-derived B-cell lymphomas and PC malignancies. This will guide the development of molecular approaches to improve antibody responses to vaccination on the one hand and therapeutic targeting of genes involved in the pathogenesis of certain autoimmune diseases and blood cancers on the other. As an example for the latter, the results may provide the basis for the development of transcription factor-targeting therapeutics such as small molecule inhibitors and proteasome-targeting approaches (PROTACs), which are projects which could be undertaken in collaboration with structural biologists. Importantly, the identification of 'druggable' targets for anti-cancer therapies may provide the basis for identifying new prognostic and diagnostic markers that allow a more refined stratification of patients with these cancers, and to inform the development of highly specific anti-lymphoma and anti-PC malignancy (e.g. multiple myeloma) drugs that could increase the efficacy of currently used treatment regimens. By focussing on identifying the mechanisms of molecular pathogenesis in autoimmune disease and blood cancer – the prerequisite of precision medicine –, the work will directly contribute to paving the basis for personalized treatment of these diseases. All outcomes of the project will be published in peer-reviewed journals and thus disseminated among the basic and clinical scientists.

Who or what will benefit from these outputs, and how?

Understanding the molecular mechanisms of the GC reaction of the humoral immune response in which long-lived PCs and memory B cells are generated is currently a major focus in the field of adaptive immunity, an area which encompasses the study of vaccination strategies as well as autoimmune disease and blood cancer. Thus, there is a large research community working on elucidating the cellular dynamics of the GC reaction, which besides B cells involves several other immune cell types. The quest to understand the basis of the GC reaction in establishing humoral immunity is expected to provide the framework for developing new ideas on how to manipulate the GC response in order to 1) enhance the antibody response to vaccination and increase durability, and 2) to control chronic inflammatory autoimmune conditions in which GC-derived PCs secrete pathogenic autoantibodies, and to 3) identify targetable vulnerabilities in GC-derived blood cancers. Therefore, in the short term, the results from our proposed project on dissecting the molecular mechanisms of the GC reaction and the development of PCs and memory B cells in the GC are directly relevant for academic researchers working in the fields of adaptive immunity, vaccinology and chronic inflammatory/autoimmune disease as well as lymphomas and PC malignancies. In addition, since many molecular mechanisms are shared among immune cell types, this work will also provide a conceptual framework for the functional analysis of other immune cell types involved in human disease, such as in T-lymphocyte subsets, which may be exploited for the development of precision medicine-based therapies.

Equally important, the results emanating from our research may spur collaborations with pharmaceutical companies which have efficient pipelines to screen for and test drugs or biologicals against putative targets, or available drugs could be repurposed for autoimmune or lymphoma therapy.

Our ultimate goal is to improve the health and wellbeing of patients, either by preventing break-through infections due to ineffective vaccination, of by inhibiting disease-causing aberrantly activated or transformed B cells. In the longer term, the insights deriving from our proposed work which through publicizing the results will have spurred translational work by us and other national and international researchers are expected to benefit patients and their carers.

How will you look to maximise the outputs of this work?

The project team including the national and international collaborators which has been assembled to dissect GC B-cell and PC development consists of the leading investigators in their respective fields, thus ensuring that the proposed study aims can be completed in the allocated time. Laboratory expertise will be disseminated through collaborations alongside internal and external seminars, in addition to providing more detailed information on methodologies to interested researchers responding to our publications (see below). We will communicate with our local collaborators through organized, regular meetings, and with our external collaborators through teleconferences throughout the project.

External dissemination of our results to lab-based academics and clinician-scientists will be achieved via peer-reviewed publications and presentations at national and international scientific meetings, including immunology and haematology meetings. Since the immunology community of the UK is well- connected and interactive, as demonstrated by the swift and effective response to the COVID-19 pandemic, there is a real possibility of a rapid clinical translation of findings resulting from basic science. The same applies to the UK lymphoma research community, with which our team is tightly connected. Overall, our research will benefit external researchers at the national and international levels, which importantly may spur additional research into the subject area using specific expertise provided by the respective research groups.

Long-term, there is potential to translate our findings on novel potential targets for pharmacological inhibition into clinical trials, since several investigators connected to us who lead, or are involved in running national clinical trials for rheumatoid disease and B-cell lymphomas/plasma cell malignancies, are interested in the development of improved treatments.

For non-academic users, we communicate our research to the interested lay audience and the charities through public/patient partnership engagement activities, laboratory tours and scientific presentations, as previously realized by the lead investigator and co-investigators. We recognise that vaccine development for infectious diseases is now in the public eye. New insights into the molecular mechanisms of long-lived PC development that may uncover novel ways of manipulating the antibody response will potentially provide new avenues for vaccine development and precision medicine-based treatment strategies in autoimmune conditions and blood cancer. Such insights are expected to lead to media coverage and dissemination to the public. Our published work will be transmitted to other media and the public through the local Press Office. In case our findings warrant wider media coverage, we will work with the media and guarantee availability of our team of investigators. Moreover, the annual public engagement events of Festival of Science organized at local or national levels provide a platform to communicate our results to schools and the public.

Species and numbers of animals expected to be used

- Mice: 2000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We plan to employ transgenic mouse models in order to better understand the molecular mechanisms that establish an effective humoral immune response on the one hand and

that can lead to the aberrant, disease-causing activation of B cells in autoimmunity and blood cancer on the other.

The mouse is the model organism of choice to study the biologically highly complex vertebrates by directed genetic manipulation, and the generation and use of transgenic mice to analyse the function of genes associated with human disease has over the last decades led to tremendous insight into the precise mechanisms of disease development and has formed the basis for a great number of clinical applications in humans. Of exceptional importance for the planned studies is the fact that the immune system of mice is well characterized and closely resembles that of the human, which has the important implication that insights from mouse models can be directly translated to humans. Indeed, the mouse and the human genome are the most highly homologous genomes of the large vertebrates. Moreover, a wealth of commercial reagents and techniques for analysing the lymphatic system of mice are available. For these reasons, this species is most appropriate for an analysis of body cells in health and disease.

Specifically, the transgenic mouse models described in this project are required for effectively recapitulating the human germinal centre (GC) B-cell response of the T cell-dependent immune response, which allows the focused study of the biological role of particular genes in the GC B-cell response.

With regards to the choice of life stages: a precondition for the planned studies is a functional immune system as it is present in adult humans. In mice, a functional immune system is established past six weeks of age. We plan to immunize mice when they are 8 to 12 weeks old.

Typically, what will be done to an animal used in your project?

Since the experiments are performed with genetically modified animals (GMAs), tissue biopsies will be taken by ear punch and subjected to molecular genotypic analysis to determine the genetic status.

Mice will be administered substances by intraperitoneal (most cases) and intravenous (rare cases) injection and/or in drinking water. Administered substances include T cell-dependent antigens (sheep- red blood cells and nitrophenyl-Keyhole Limpet hemocyanin) with or without adjuvants (Complete or Incomplete Freund’s Adjuvant) and substances that label proliferating cells (e.g. BrdU, EdU). For most experiments, one animal may receive a maximum of 2 different substances in a maximum of 3 administrations repeated 2 to 42 days after initial immunization.

Blood (50µl) may be taken from a superficial vessel, e.g. tail vain 7 days before the first immunization and with 7-42 days interval after the first immunization and then throughout the experiment. The purpose of the blood withdrawal is to determine the titer of model antigen-specific antibodies in the serum that are generated during the T cell-dependent immune response.

Typical experiment A: a mouse is immunized with a T cell-dependent antigen and then humanely killed for analysis of the lymphoid organs 10 to 14 days later.

Typical experiment B: a mouse is immunized with a T cell-dependent antigen and then 21 days later the immunization is repeated, before it is humanely killed for analysis of the lymphoid organs 10 to 14 days later.

Blood may be taken in A and B 7 days before the first immunization and 7 to 14 days after the first immunization.

What are the expected impacts and/or adverse effects for the animals during your project?

Immunizations with the T cell-dependent antigens sheep red blood cells (SRBC) and nitrophenyl- Keyhole Limpet hemocyanin (NP-KLH) in Freund's Adjuvant are at the centre of this project. In a low percentage of mice, expected adverse effects are mild inflammation with oedema and erythema (granulomatous peritonitis) at the site of SRBC and antigen-in-adjuvant administration. Freund's Adjuvant is chosen as adjuvant because in combination with the NP-KLH model antigen it gives the best antigenic response as measured by germinal centre (GC) induction. Moreover, by using Freund's

Adjuvant, repeatability of previous work is ensured, since in the past this adjuvant has been used in a large number of studies, including ours. This will allow to assess and compare our results from the proposed immunization experiments on the background of published studies.

Mice displaying an exaggerated response to the induced inflammation will be monitored daily by the animal technican and advice sought from the NVS when erythema and pustule formation is associated with lethargy due to hyperthermia; where necessary mice will be removed from the study and culled promptly by a humane killing method. Furthermore, the body condition of mice will be monitored and any mice culled if they show signs of ill health such as piloerection / hunched posture / lack of appetite for a period of 48h.

No adverse effects are expected to be associated with blood sampling or administration of BrdU or EdU.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Protocol 2 falls under the category moderate severity since a fraction of mice may develop mild inflammation with oedema and erythema at the site of antigen administration. The percentage of immunized animals displaying an exaggerated response to the induced inflammation is estimated to be lower than 5%.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of this project is to understand complex biological processes, most notably including the molecular mechanisms of the T cell-dependent antibody response which generates high-affinity plasma cells (PCs) and memory B cells, in primary cells in vivo. This information is expected to be directly relevant to the development of vaccination-enhancing compounds as well as novel anti- inflammatory and anti-blood cancer therapies. Regarding this particular project it is important to note that the development of PCs and memory B cells occurs in the germinal centre (GC), which is a specialized microenvironment which develops in T cell-dependent immune responses upon antigen administration in the spleen and lymph nodes. The GC B-cell response involves complex molecular processes that contribute to the affinity maturation of the immune response as well as to the generation of immunological memory; most important to note here is the process of somatic hypermutation of the antibody genes, which is a DNA-modifying mechanisms (and thus a potentially mutagenic process occasionally giving rise to B-cell cancers) that together with selection mechanisms generates antibodies with improved binding to the disease-causing pathogens.

So far, it has been impossible to reproduce the GC microenvironment in vitro, which is due to the fragile nature of these cells that do not survive in culture (as documented by extensive literature), as well as to the complex composition of this microenvironment, which includes various amounts of B cells, T cells, follicular dendritic cells and macrophages that require the activity of multiple cytokines and chemokines for their function and survival. None of these complex individual events occurring in the living organism over a large time-window are available via other cellular systems.

Which non-animal alternatives did you consider for use in this project?

For the gene/pathway identification studies, we have considered cell culture systems that co-culture human tonsillar GC B cells on stromal cells (organoids), which are being developed with the aim to mimic the GC reaction occurring in vivo.

For some aspects of our study regarding the gene/pathway validation studies as well as e.g. the investigation of the deregulated function of proto-oncogenes, whenever possible we plan to investigate those at the molecular level using appropriate in vitro cultures of human cell lines.

Why were they not suitable?

Until now, no functional in vitro cell culture system has been developed that faithfully mimics all aspects of the in vivo GC reaction. A major caveat is that the in vitro cultured cells do not undergo somatic hypermutation, and the requirements for selection of high-affinity B cells that include a specific subset of T cells and follicular dendritic cells are as of yet impossible to mimic in the culture. Thus, in the absence of an in vitro system for the highly complex process of GC B-cell development, the use of animal models remains the only rational approach to study their role in the context of the complex living organism.

Another limiting factor of using human B cells is that in our proposed project, we aim at the identification of very small GC B-cell subpopulations that become visible and thus identifiable only through the expression of a living-color fluorescent marker protein which has been targeted into the mouse genome so that it is expressed in the GC B cells of interest. Indeed, a major aim of the project is to unambiguously identify and characterize these functionally defined GC B-cell subsets in mice by benefitting from the possibility of targeting the mouse germline, which will provide information to identify the corresponding cell subset among human GC B cells.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our previous experience documented by relevant publications [see Experience section below] allows us to ensure that animal usage is kept to a minimum with experiments designed such that we expect the outputs to be measurable and reproducible.

In order to achieve statistical significance and experimental reproducibility, we use 5-10 mice per genotype group. For the experiments involving immunization, 5 mice per experimental point is the commonly used number to obtain interpretable, statistically significant results, as extensively documented in the scientific literature. For example, in the experiments utilizing flow cytometry, measurement of newly identified markers, assuming a 20% change in samples between two genotypes, with an SD of 10%, gives 5 mice per group for an alpha level of 0.05 with 90% power when using a 2-sided Student's t-test. This calculation thus represent the optimum number of animals needed to attain statistical significance of $p<0.05$ with a 90% probability. Because of the nature of the immunization experiments, outlayers can occur e.g. due to inefficient immunization (documented by studies), thus requiring sample sizes of more than 5 mice, with the actual number depending on the outcome (phenotypic difference among the genotypes). For

functional studies on B cells, due to the limited number of B cells one can obtain from a mouse, appr. 10 mice per group are required for functional in vitro studies (e.g. assays for activation of biochemical signalling pathways and cell differentiation) on isolated B cells aimed at understanding the molecular consequences of the B cell- specific deletion of the signalling molecules and transcription factors.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The numbers of mice to be maintained and used in experiments will be kept at a minimum to ensure that reliable experimental data is obtained (see power-calculation example in Figure 2 of Protocol 2 in the section "How will you determine group sizes"). To achieve this, we based our group size determination and randomization on the vast literature of immunological research with animal models, in addition to implementing the Experimental Design Assistant (EDA) from NC3Rs in our study design. Moreover, we follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines to ascertain that our research design is compatible with the ARRIVE checklist of recommendations. A potential sex bias in the resulting data is eliminated by using both male and female mice.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our project includes efficient breeding to reduce the number of GMAs that are not of the desired genotype, which is a general caveat of the interbreeding of multiple alleles. For example, in the experiments with the experimental cohort of geneXfl/flgeneY-Cre (tissue specific knockout), geneXfl/+geneY-Cre and geneX+/+geneY-Cre, we follow a breeding strategy in which geneXfl/fl mice are intercrossed with geneXfl/+geneY-Cre mice to obtain Cre-expressing homozygous and heterozygous mice for the floxed allele, and geneX+/+geneY-Cre with geneX+/+ mice to obtain the Cre- expressing control mice. This strategy, which is possible to follow since all mouse lines are on the C57BL/6 genetic background, considerably reduces the generation of ‘unwanted’ genotypes that would have to be humanely culled.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

As a general consideration which is highly applicable to refinement: the mouse is the model organism of choice to study the biologically highly complex vertebrates by directed genetic manipulation, and the generation and use of transgenic mice to analyze the function of genes associated with human disease has over the last decades led to immense insight into the pathogenetic mechanisms of disease development and has formed the basis for a sizable number of clinical applications in humans. The endogenous mouse genome can be manipulated in mouse embryonic stem (ES) cells, which limits the use of animals in this phase (i.e. by not having to identify and select naturally occurring mutants).

The project entails minimal, momentary pain and distress associated with genotype identification, intraperitoneal (i.p.) and intravenous (i.v.) injections, blood withdrawal, and euthanasia. Immunization by i.p. injection may occasionally cause localised inflammation with oedema and erythema (granulomatous peritonitis), which however does not necessarily lead to chronic distress. Mice will be monitored daily for general health status following i.p. immunization.

The severity limit for most mice will be mild. Specifically, the majority of animals produced under the breeding program will be used to supply tissues for in vitro and lymphoid tissue analyses (flow cytometry, immunohistochemistry). The mouse lines used in the project have no defects beyond alterations in or loss of immune cell populations and suffer no ill effects in an SPF animal facility. The low percentage of mice displaying an exaggerated response to the induced inflammation due to immunization with the T cell-dependent antigens sheep red blood cells (SRBC) and nitrophenyl- Keyhole Limpet hemocyanin (NP-KLH) in Freund’s Adjuvant (moderate severity) will be monitored daily for signs of ill health such as piloerection / hunched posture / lack of appetite and advice sought from the NVS; where necessary mice will be removed from the study and culled promptly by a humane killing method. Immunizations with SRBC and model antigens such as NP-KLH in adjuvant are currently the most refined methods to study the T cell-dependent immune response in mice. These immunization models are well established and are required for immunological research, and in addition allow the comparison of results from immunization experiments among different research groups. No adverse effects are expected to be associated with blood sampling or administration of reagents to determine proliferation (e.g. BrdU).

For all procedures, we will apply the least invasive methods of dosing and sampling appropriate to the objectives of the experiment, including the use of anaesthesia for humane restraint where necessary.

Why can’t you use animals that are less sentient?

With regards to the life stage: a precondition for the planned studies is a functional immune system as it is present in adult humans. In mice, a functional immune system is established only past six weeks of age. Thus, by studying the immune system of adult mice, we ensure that our findings are relevant to adult humans.

The adaptive immune system as found in humans, a complex system of cells and organs that recognize, destroy and memorize invading pathogens, has evolved only in higher vertebrates. Of note, the mouse and the human genome are the most highly homologous genomes of the large vertebrates, and the immune system of mice is well characterized and closely resembles that of the human, with both being different from other species as e.g. fish and birds (chicken).

With regards to animals that have been terminally anaesthetized: this option is not applicable as the project does not include the highest severity level.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In our facility, animals are monitored daily and their health status is recorded using an established animal welfare scoring system. After i.p. injection of substances, animals are closely observed for adverse effects, and monitored daily for signs of ill health such as piloerection / hunched posture / lack of appetite.

We have established a protocol for the use of Freund’s adjuvant but will also actively seek to replace this adjuvant with less invasive substances for the emulsification of antigens, should these become available during the project, by conducting small scale pilot studies and comparing results with our established protocol.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the most recent Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, which are currently The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published in PLoS Biol 18:e3000410, 2020; PMID: 32663219; PMCID: PMC7360023.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will make use of the NC3R website and available 3R online resources. Through our connection with other local and national research groups, we have the opportunity to discuss protocol-relevant issues that may benefit our protocols.

We will closely follow any new developments in the area of cell culture systems that co-culture human tonsillar GC B cells on stromal cells (organoids), as advances may allow to study some aspects that we currently plan to investigate in animals by implementing organoids. To this end, we will follow newly-published research (including Bioarchive or Medarchive servers) and screen abstracts of national and international conferences.

We participate in events organized by the local Animal Welfare Ethical Review Body (AWERB), which also informs us promptly about any new developments in the field.

149. Cellular and Acellular Control of Cochlear Function

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cochlea, Hearing physiology, Hearing loss, Hereditary deafness, Hearing restoration

Animal types	Life stages
Mice	adult, neonate, juvenile, pregnant
Guinea pigs	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The major aim of this research proposal is to understand the complex electromechanical functional relationships between cellular and extracellular elements of the organ of Corti in the mammalian cochlea through recording of responses from normal cochleae and cochleae with modified expression of specific proteins. Another aim is developing an efficient method for drug delivery along the entire extent of the cochlea.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Understanding the complex in vivo interaction between the sensory hair cells, supporting cells and tectorial and basilar membranes is essential for the future development of successful treatments for hearing loss, especially those involving recovery of damaged, or replacement of, dead sensory hair cells. Because the cochlea is one of the most difficult organs for drug delivery, local drug administration into the middle ear is routinely used. This local administration, however, leads to formation of large drug concentration gradients along the length of the cochlea. We have developed a method of cochlear pumping which enables efficient drug delivery throughout the cochlea. Development of the method will allow efficient current and future treatment of hearing disorders.

What outputs do you think you will see at the end of this project?

The project will result in a number of publications in high profile journals, new knowledge and improved protocols for cochlear drug delivery, and a prototype device for delivering drugs along the entire extent of the cochlea.

Who or what will benefit from these outputs, and how?

Hearing loss is one of the most common disabilities in the world. The Royal National Institute for the Deaf estimates that one in five UK adults suffers some form of hearing loss. While hearing loss has many causes, including noise damage, age, drug toxicity and genetic factors, the shared impact of these etiologies is increased social isolation, depression and risk of comorbidities such as dementia. Beyond individual health impact is the knock-on economic cost of hearing loss – the UK Health and Safety Executive estimates that as many as 2 million British people are exposed to unsafe noise levels at work, resulting in noise induced hearing loss being the second most common reason to claim employer’s liability insurance. Therefore, understanding the cochlear working, which includes understanding of functional relationships between cochlear cellular and extracellular elements, is an important field of research. Our research publications will immediately increase understanding by the research community on how cellular and non-cellular elements interact and contribute to the workings of normal and impaired cochleae. In the longer term, adoption of our methods of drug delivery by clinics should enhance the efficacy of treatment of hearing disorders and facilitate development of new approaches to dealing with hearing dysfunction.

How will you look to maximise the outputs of this work?

We have developed a strong international collaboration across academia and with industry in the UK. We shall continue this collaboration to ensure the most productive work and quickest adoption of our findings. Further dissemination of the new knowledge generated during the project will be achieved through presentations at major international meetings.

Species and numbers of animals expected to be used

- Mice: 3000
- Guinea pigs: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The guinea pig cochlea has been used extensively as a model of the human cochlea, especially in research on cochlear drug delivery. The guinea pig cochlea is much larger than the mouse cochlea which makes possible experimental interventions (e.g. microelectrode and laser interferometric recordings) which are not possible in mice. However, mice are the most appropriate animals to use for experiments on modifications of cochlear specific proteins. A number of methods to produce genetically modified mice has been developed. We have developed special techniques to make mechanical measurements from the cochlea of mice.

Typically, what will be done to an animal used in your project?

The project includes breeding of genetically modified animals that have been developed by our collaborators. The project is not expected to produce any harmful phenotypes because we target only specific proteins of cell types within the cochlea itself that modify hearing function and do not result in total deafness. All the other procedures, except the exposure to moderately loud sounds (up to 90 dB of Sound Pressure Level re 2 x 10-5 Pa), will be carried out using anaesthesia which will be induced via intraperitoneal injections. In all cases, except those employing non-invasive, non-traumatic assessment of auditory function (measurement of distortion product otoacoustic emissions), the animal will be euthanized by anaesthetic overdose at the end of the procedure.

What are the expected impacts and/or adverse effects for the animals during your project?

No marked adverse effects are expected to impact animals during the project. Given the importance of hearing in the mouse, some abnormal behaviour from mutant mice may occur due to alterations in their hearing function. However, the strain of mice we are using feed and breed normally and the mutations do not have marked impact on animal welfare.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Only non-recovery procedures will be used for guinea pigs. The maximal severity expected for mice is moderate and it is associated with breeding of genetically modified animals without harmful phenotypes, animal recovery from light anaesthesia and exposure of awake mice to moderately loud sounds (up to 90 dB of Sound Pressure Level re 2 x 10-5 Pa).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals have to be used for these experiments because the study of detailed interaction of its cellular and extracellular components in the function of the normal and genetically modified (including genetic disorders) cochlea is the purpose of the project. In vitro hearing organs are not yet available. Any attempts to isolate the cochlea for in vitro study would compromise its normal function and would result in severe increase in thresholds of its neural and mechanical responses.

Which non-animal alternatives did you consider for use in this project?

We shall continue to use computational modelling of the workings of the cochlea to gain additional insight into mechanisms of mammalian hearing and to minimize the number of animals used.

Why were they not suitable?

Computational modelling is only effective if it is based on and validated by experimental data. This reiterative process of modelling and testing is essential, otherwise our research outcomes would become speculative and not reflect the true cochlear function. A major benefit of combining computational modelling with animal experimentation is that it greatly reduces the number of animals used.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimate is based on average number of animals reported in Annual Return of Procedures for years 2016-2019 (~2,000 mice). It also takes into account that guinea pigs will be used for two separate projects, namely, to study cochlear physiology and anatomy and a translational project on developing a method for efficient drug delivery to the cochlea.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

ARRIVE guidelines are followed while planning the experiments. Our experiments are technically challenging, but we have a high success rate of > 80%. The essential feature we are looking for is repeatability of measurements. We can usually achieve this objective in measurements from 10 -20 preparations to give us repeatable measurements from 5 – 10 preparations that fall within the 95% confidence limit of a mean. Post hoc analysis is made after each experiment to minimize the number of animals used. Preparations that fail this test do so because of surgical errors made during preparation (very rare) and physiological failure, which result in a limited data set.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Because the mammalian cochlea is not readily accessible and because experimental manipulations to the cochlea are technically challenging, our experimental data are used to develop and refine computer models of the cochlea. Insights gained from simulation of cochlear responses using these models will allow for reduction of animals used in future experiments. We normally share animals for in vivo and in situ experiments. After initial data collection in vivo, the same animals are killed by a Schedule 1 method and used for in vitro recording from single cells in isolated cochlear preparations.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The guinea pig cochlea has been used extensively as a model of the human cochlea, especially in research on cochlear drug delivery. The guinea pig cochlea is much larger than the mouse cochlea which makes possible experimental interventions (e.g. microelectrode and laser interferometric recordings) which are not possible in mice. However, mice are the most appropriate animals to use in experiments when genetic modification of cochlear specific proteins is required. We have developed special techniques to make mechanical, electrical and acoustic measurements from the cochlea of deeply anaesthetised mice and guinea pigs. Hence any suffering and distress to the animals is excluded. The genetic manipulations to the cochlear specific proteins, we will be using, do not result in harmful phenotypes.

Why can’t you use animals that are less sentient?

All procedures will be carried out using anaesthesia. In all cases, except those employing non- invasive, non-traumatic assessment of auditory function (measurement of an echo from the ear in response to sound stimulation), the animal will be killed by anaesthetic overdose at the end of the procedure.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All our breeding animals and litters are monitored on daily basis by experienced animal technicians and personal licence holders to identify animals experiencing discomfort/pain. All the animals which show these conditions above that expected in a breeding colony and during protocols will be immediately culled. We use the ear clipping preferred method for utilising tissue because it is less invasive than other methods and combines 2 protocols (ID and genotyping) thus complying with the 3Rs directive to minimise suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

ARRIVE guidelines are closely followed to ensure our experiments are optimized and are conducted in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are subscribed to the NC3Rs newsletter and regularly attend relevant webinars and meeting organized by the NC3Rs. Regular meetings are organized with invited external speakers on different aspects of animal research.

150. Development and Use of a Large Animal Vascular Aneurysm Model for Testing Translational Interventions

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

aneurysm, aorta, grafts, stents, hypertension

Animal types	Life stages
Minipigs	adult, juvenile
Pigs	Juvenile

Animal types	Life stages
Sheep	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop a large animal vascular aneurism model and to use it for the preclinical safety and efficacy assessment of novel diagnostic and therapeutic interventions intended for use in humans.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

A vascular aneurysm is the ballooning of a blood vessel at a site of localised weakness. They are commonly linked to the development of high blood pressure and are the second most common of all vascular diseases. Vascular aneurysms usually form in a major artery, particularly around a site of branching. The aorta is especially prone to the developments of aneurysms, such that in the UK, 1.5% of men and 1 % of women will develop an aortic aneurysm by the age of 65. Most aneurysms are asymptomatic, so usually patients are unaware of their development. Once an aneurysm has started to form, it will continue to expand progressively until it ruptures, often with fatal consequences. In the UK, 1 in 75 deaths in those over 65 years of age is caused by the rupture of an aneurysm. Whilst the majority of aneurysm occur in the eldely they also feature frequently in babies suffering with cardiovascular defects. At present there is no effective medication for vascular aneurysms and treatment is reliant on either endo-vascular or open surgical repair, neither of which are curative and both of which carry a risk of complications. Endo-vascular repair is unsuitable for the treatment of an aneurysm at a branch point in a vessel and current designs have a high risk of leakage, necessitating secondary repair. Open surgical repair involves the grafting of tissue onto the vessel wall and carries a significant risk of surgical complications. A number of small animal models of aneurysm have been described however, these are unsuitable for the assessment of novel diagnostic and therapeutic interventions intended for use in a clinical setting. The purpose of the outlined studies is to develop a large animal aneurysm model and to use it to assess new imaging and interventions for the treatment of this condition.

What outputs do you think you will see at the end of this project?

By the end of the study, it is to be expected that a large animal model of vascular aneurysm will have been developed and a description published in a peer reviewed medical journal. Using the developed model, data will be generated on the efficacy of novel vascular grafts and stents for use in the treatment of vascular aneurysms, thereby facilitating their translation into a clinical setting. The data generated during the study will advance understanding of how aneurysms develop and determine the effectiveness of novel interventions aimed at either, slowing or halting their progression or restoring normal vascular architecture.

Who or what will benefit from these outputs, and how?

In the short term, the development of a large animal model of vascular aneurysm will be of benefit to scientists and clinicians working to develop more effective treatments for this condition. In the medium term, the work will benefit companies developing novel interventions for the treatment of vascular aneurysm by generating the data required by regulators to translation the treatment into first in human clinical trials. In the long term, the work is expected to be beneficial to both patients and clinicians by speeding the translation of improved treatments into the clinical setting.

How will you look to maximise the outputs of this work?

This project will be conducted in collaboration with medical experts in the treatment of vascular aneurysms: the findings will be disseminated in presentations at medical conferences and through publications in scientific and medical journals.

Species and numbers of animals expected to be used

- Pigs: 78
- Minipigs: 72
- Sheep: 72

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The study will use pigs, sheep and mini-pigs. These species have been selected because the size of their aorta, a major site of vascular aneurysm, is sufficiently similar to that of humans (pigs and sheep:

adult humans, mini-pigs: babies) to enable devices and interventions intended for translation into a clinical setting to be evaluated. Juvenile pigs will be used for short term studies, as an abdominal aortic aneurysm models has already been described in this species. Adult sheep and mini-pigs will be used for long term studies as they are no longer growing and are therefore able to provide a more representative model for such studies. Adult sheep will be used for devices intended for adult humans and mini-pigs for devices intended for the treatment of babies.

Typically, what will be done to an animal used in your project?

Following arrival on the unit, the animals will be habituated to human company for a least five day before any regulated procedures are undertaken. The animal will then undergo a minor surgical procedure, which may be performed on up to four occasions under general anaesthesia, to induce a vascular aneurysm. Surgery may include the insertion of a minipump under the skin to deliver drugs known to predispose to the development of

vascular aneurysm and/or the distention of a blood vessel using a balloon catheter inserted into a peripheral vessel and guided into position with the aid of non- invasive imaging. All animals will be given per-operative pain control, which will be maintained until the animal shows no discernible signs of pain. All animals are expected to make an uneventful recovery from surgery and to resume normal behaviour within a few hours. The induced vascular aneurysm is not expected to have any detrimental effect on the wellbeing of the animal throughout the duration of the study.

Subsequently the animals will be periodically anaesthetised and imaged to determine the extent and size of the aneurysm.

Animals with an established vascular aneurysm may undergo a procedure to restore the normal integrity of the vessel. This may include drug treatment, the insertion of an endo-vascular device or the surgical placement of a patch or graft. In all cases, the repair procedure will mirror that intended for use in humans. All animals undergoing surgery will be given drugs to control post-operative pain, which will be maintained until the animal shows no discernible signs of pain. All animals undergoing surgery are expected to make an uneventful recovery and to resume normal behaviour with a few hours.

What are the expected impacts and/or adverse effects for the animals during your project?

All animals are expected to experience some mild pain during the immediate post-operative period however, this is not expected to persist beyond a few days and all animals are expected to resume normal behaviour within a few hours of recovery. All animals will be given post-operative pain control until they show no discernible signs of pain. All surgical procedures carry a risk of haemorrhage, however the surgeons performing these are highly experienced NHS specialist cardiovascular surgeons and the animals will be given fluid replacement to ensure that any blood loss does not adversely affect their wellbeing. All surgery carries a risk of infection however, this will be minimised by using aseptic precautions, in-line with that used within the NHS and consistent best surgical guidelines. When considered necessary, prophylactic antibiotics may be given to minimise the risk of post- operative infections.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate, all animals.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The purpose of the outlined work is to develop a large animal vascular aneurism model and to use it for the preclinical safety and efficacy assessment of new medications, intravascular devices and surgical patches or grafts for the treatment of vascular aneurysm's. The ultimate aim of the work is to produce the data needed to translate successful developments into human clinical trials. It is necessary to use animals for these studies as only data generated using a representative animal model will suffice to meet the requirements of the regulators responsible for authorising such trials.

Which non-animal alternatives did you consider for use in this project?

Whilst ex-vivo models of aortic aneurysm have been described, including the use of human umbilical cord tissue, they are unsuitable for assessing the efficacy and safety of medical devices and intervensions as the duration of tissues integrity is very limited . Furthemore, only data produced using a representative animal model will suffice to meet the requirements of the regulators responsible for authorising the translation of novel developments into first in human clinical trials.

Why were they not suitable?

Non-animal alternatives are not suitable for these studies as only data produced using a representative animal model will suffice to meet the requirements of the regulators responsible for authorising the translation of novel developments into first in human clinical trials.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimate for the number of animals required for these studies is based on my previous experience working on similar cardiovascular research projects and the informal level of interest expressed by biomedical companies developing products for the treatment of a vascular aneurysm.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The criteria needed to produce a representative animal model for the outlined studies have yet to be fully determined consequently, the initial studies will focus on this aspect. Data generated during these initial studies will subsequently be utilised for the study controls, thereby minimising the number of animals needed. In addition, this initial data will be used in power calculations to determine the group size needed for the studies aimed at assessing the safety and efficacy of interventions and devices intended for the treatment of vascular aneurysm. Where appropriate, studies will be designed using the NC3R's Experimental Design Assist software or a comparable computer program.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The data generated during the initial phase of the study, aimed at determining the criteria needed to produce a representative animal model, will subsequently be used for the study controls thereby minimising the number of animals needed. In addition, this initial data will be used in power calculations to determine the group size needed for the studies aimed at assessing the safety and efficacy of interventions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The study will use pigs, sheep and mini-pigs and will involve the induction of a vascular aneurysm and its subsequent repair . The aneurysm will be induced using a minimally invasive procedure performed under general anaesthesia. Upon recovery, the animal will be given drugs to control pain until it shows no discernible signs. All animals are expected to make an uneventful recovery from the procedure and to resume normal behaviour within a few hours. The induced aneurysm is not expected to cause pain or impair the wellbeing of the animal. The aneurysm will be monitored periodically by non-invasive imaging performed under general anaesthesia. Once the aneurysm is established, a repair procedure will be performed under general anaesthesia in-line with that used within the NHS but using novel drug treatments regimes, endo-vascular devices, grafts or patches. Upon recovery the animal will be given drugs to control pain until they show no discernible signs. All animals are expected to make an uneventful recovery from the procedure and to

resume normal behaviour within a few hours. Animals will be maintained for a pre-defined period, during which they may undergo further non-invasive imaging under general anaesthesia. At the end of the study the animal will be killed under terminal anaesthesia.

Why can’t you use animals that are less sentient?

The purpose of the outlined work is to evaluate novel interventions for the treatment of vascular aneurysm in humans with the aim of facilitating the rapid translation of promising approaches into human clinical trials. To this end, it is essential that the model system used closely replicates the human scenario. Only a medium sized mammal can meet the criteria of size, anatomy and physiology required by the regulators authorising human clinical trials, therefore it is not possible to use less- sentient animals for these studies. Short term studies will be conducted in juvenile pigs however, this species is unsuitable for long term studies as they continue to grow. Consequently, long term studies will be performed using either mature mini-pigs or sheep. Sheep will be used to assess interventions and devices intended for use in adult humans whilst minipigs will be used for those intended for pediatric patients.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedures used will be performed in a manner that closely replicates that used in the treatment of vascular aneurysm in the NHS. I am an experience NHS specialist cardiovascular surgeon and, in addition, have extensive experience performing cardiovascular interventions on pigs and sheep under other PPLs. In all cases the procedures used will be refined to minimise, as far as possible, any tissue damage and will be performed using the same aseptic precautions as used with the NHS. All animals will be provided with pain control, under the guidance of a specialist veterinary anaesthetist, comparable to that given to human patients undergoing such procedures. Post-surgery, animal care will be provided by specialist animal technicians experienced in the post-surgical management of pigs and sheep.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All surgical procedures will be performed in line with best NHS practice and using standards that meet or exceed those described in LASA guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

My institute host regular 3Rs meeting and has a NC3Rs representative who work closely with the NIO to ensure that all animal users are kept up to date with the latest advances. In addition, I am keen to work with the NACWO to ensure that, as far as possible, the animal

needs are fully met and that all procedures are refined to optimise the animals experience throughout the study.

151. Identification of Interventions to Maintain Health and Resilience With Age

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Ageing, Multi-morbidity, Frailty, Geroprotectors

Animal types	Life stages
Mice	juvenile, adult, aged, pregnant, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to identify and test which geroprotector promotes health, prevent age- related diseases and boosts resilience with age.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Age-related multimorbidity (two or more chronic diseases) affects over 50% of those over the age of 65 and is responsible for over 50% of GP consultations. Currently, each disease is treated individually leading to polypharmacy, increased adverse effects, and reduced efficacy. Frailty defined as an accumulation of deficits and reduced ability to respond to adverse events (loss of resilience) affects 25- 50% of those over the age of 80, the fastest growing segment of the population. Frailty often leads to loss of independence. A patient with frailty costs double of one of similar age without frailty. With an increasing ageing population new cost-effective ways to prevent multimorbidity and frailty are required to ensure sustainable healthcare costs.

Ageing is the major risk factor for multimorbidity and frailty. Research in the last 10 years shows that it is malleable, and it can be decelerated by exercise, specific diet regimens and drugs, which interferes with specific mechanisms of ageing such as DNA damage, mitochondrial dysfunction, and senescence. These drugs are called geroprotectors. By interfering with mechanisms of ageing geroprotectors have been shown to decelerate biological ageing and prevent or reduce the severity of multiple age-related diseases and frailty in mice. For this reason, they hold potential in the prevention of multimorbidity and frailty.

Whilst progress have been made there are still important questions that need to be answered before these drugs can realise their full potential.

1. Are there more powerful geroprotectors or health practises regimes, which may be administered only for short periods of time that can delay or reverse ageing?

2. Do they promote the health of every tissue or each geroprotector improve the health of selected tissues depending on the mechanisms of action?

3. What is the relationship between tissue ageing and disease development?

4. What is the relationship between diseases which may cluster together? Does one accelerate the other?

5. Can we develop geroprotectors or health practises regimen, which target multiple diseases? Are they cluster specific?

6. Can geroprotectors improve frailty and be used to boost resilience in older patients undergoing an adverse event?

What outputs do you think you will see at the end of this project?

We will have generated knowledge of whether interfering with mechanisms of ageing such as DNA repair and senescence improve healthspan by preventing tissue ageing, frailty, and diseases (e.g., atherosclerosis and osteoarthritis)

We will have developed knowledge as to whether there is a common ageing mechanism driving multiple diseases (e.g., atherosclerosis and osteoarthritis)

We will have disseminated this knowledge by publishing 1-2 papers in peer reviewed journals

We will have advanced 1-2 drugs and generated a data package to support their testing in a clinical trial to boost resilience in older people

Who or what will benefit from these outputs, and how?

We will understand whether interfering with mechanisms of ageing improves tissue health and make tissues more resistant to develop disease. This knowledge will be important to influence policy makers to fund more of this research so that progress can be faster. This will also provide the evidence for clinicians and industry to change the way they think about treating diseases of older age and encourage them to test these interventions in patients, once the appropriate preclinical studies have been performed. Finally with these drugs older people can stay healthy for longer, reducing the costs of health and social care resulting in a better quality of life.

How will you look to maximise the outputs of this work?

This work will be performed collaboratively, and the outputs disseminated through publications and presentations at conferences. Some of this work may add strength to the applicant's work to persuade industry, the regulators, policy makers, funders, and the patients about the added value of geroprotectors. As they are a new class of drugs, which target a population of patients who is usually excluded from clinical testing, much knowledge is required to develop appropriate clinical protocols.

For this to happen strong preclinical data are required. It also requires a new framework for implementation in the health system as they are preventive and target more than one disease. Through the Healthy Lifespan Institute website, newsletters to stakeholders and public engagement events we will disseminate the knowledge developed to a wider audience. We will organise events with policy makers and research funders to influence the creation of funding to support the clinical translation of geroprotectors and forge a path for implementation of these drugs in routine care.

Species and numbers of animals expected to be used

- Mice: 600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the most commonly used animals to study regeneration and ageing and for drug testing. This means we have lots of information about them, which allows us to design better experiments. We will use extensive in vitro testing before animal testing. Only the most promising drugs will be tested in animals. We will use mice from 4 weeks of age up to 30 months. Ageing is a process that occur throughout the life of the organism. For this reason, it is important to study the health of the animal throughout their lifespan. In addition, ageing is the major risk factor for age-related diseases and yet often animal models do not account for this important factor. For this reason, we will use age-appropriate animals when studying diseases, which better reflect what happens to patients.

Typically, what will be done to an animal used in your project?

Animals may harbour genetic modifications or may be exposed to factors (e.g., high fat diet), which accelerate or decelerate their ageing and frailty or that will induce up to two age-related diseases to mimic multimorbidity. They may be given a drug to decelerate their ageing and examined to see if they stay healthier by assessing the function of the main tissues such as the heart, the muscle, the brain.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect adverse events for most procedures. Occasionally the animals may be sick (similarly to cancer patients undergoing radiotherapy or with age). They may display diarrhoea, infections, weight loss and problems of mobility. With advancing age mice accumulate multiple health problems similar to older people. If the effects are too severe, we will humanely kill the animals. At the end of the experiment the animals will be humanely killed, and their tissues examined and analysed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We will only use mice. Approximately 30% of the protocol are expected to have a mild severity and the remaining are moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There is no in vitro test which can assess the complex effects that ageing has on multiple tissues and they have on each other. For example, we know that the health of the intestine or of the immune system can influence the activity of other tissues such as the brain. In addition, testing of the efficacy and safety of drugs, or how much of the drugs reach the interested tissues can only be done in a living organism with a circulatory system.

Which non-animal alternatives did you consider for use in this project?

We culture and age cells in our laboratory to study some of the effects of ageing on individual cell populations. These tests always precede the use of animals. If we see promising effects in decelerating ageing, we progress the testing using Drosophila, a non-vertebrate model, which recapitulate the effects that fundamental mechanisms of ageing have on healthspan and lifespan. Only those molecules which will show effects in Drosophila will be tested in mice. Our group has attempted to make a model of ageing within zebrafish, but the data is currently inconclusive though we also work closely with another group on their complementary approach of a zebrafish telomerase model. In addition, our group is working with engineers to create computer models, which mimic the 3D tissue structure and the relation among the different structure, like a digital mouse. It is hope that, in the future we will be able to perform some of the experiments, which requires animals by using computer models.

Why were they not suitable?

Studies using cells do not reproduce the 3D environment of a living organism. Drosophila is a non- vertebrate model with well conserved mechanisms of ageing. However, their biology is very different from that of mammalian organisms. Computational models are at the very early stages of development and not ready to be used for testing.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For healthspan assessment we will use N=20/group. This has been calculated based on previous experience and following a consensus process we have performed with other laboratory worldwide during which we have standardized the protocols and all the

variables as much as possible to reduce the number of animals and to enable comparisons of data across laboratories (see Bellantuono et al

Nat Protoc 15, 540–574 (2020)). As there are several protocols, we have based the estimation of the number of animals on the test with the highest variability (e.g., grip strength can be operator dependent and has high variability) and based the size of the effect on the best data available where a positive effect was found using this test. As there is no data in patients, we do not know yet how biologically significant this is going to be. For other experiments we will need to perform pilot studies to establish the appropriate number of animals. We also need to add 20% more mice to account for the attrition due to the fact that some mice will be lost as part of the ageing process.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have in place a pipeline where drugs are tested in cells first, then in a non-mammalian model (Drosophila or Zebrafish larvae) and only those molecules with significant effects on healthspan and lifespan can progress to testing in mice. We have worked with other laboratories worldwide to ensure the experimental design was state- of-the-art (See Bellantuono et al. Nature protocol 2020), we had selected the least invasive technique able to test function of the different organs and we performed the procedure according to best practise and we have appropriate humane endpoints when dealing with ageing animals.

During the duration of the licence, before each experiment is conducted, a detailed protocol will be written covering: (i) a statement of the experimental objectives; (ii) a description of the experiment, covering such matters as the experimental treatments, the size of the experiment, and the experimental material; and (iii) an outline of the method of analysis of the results (iv) a detailed plan of the personnel involved, including who performs the randomization, who is blinded and will perform the tests. Factorial designs are preferred, and power analysis is used where appropriate. We will take advice from experimental design specialist where appropriate

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have detailed the approach above. In addition, with the development of computational approaches to the analysis of musculoskeletal health we will be able to obtain more accurate measurements which will reduce the number of animals. For example, we have developed new computational methods for the analysis of bone structure following intervention, which is based on longitudinal imaging by microCT and automatic image registration and this has reduced the number of animals by 63%. We have also developed a computational approach to measure bone strength. Whilst in the past this required the humane killing of the animals now, we are able to do this analysis in silico based on the microCT images collected when analysing bone structure.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Most knowledge in the field of ageing for mammalian systems has been developed in mice and they are the most commonly used species accepted by regulatory bodies when testing new drugs for proof- of-concept studies. We will use methods to accelerate the events occurring during ageing such as using radiation, manipulation of diet or gonadectomy, or to induce specific diseases such as osteoarthritis, osteoporosis, and cardiovascular diseases. We will also use methods to test the effects of drugs decelerating ageing on healthspan. All our protocols are mild or moderate in severity.

Why can’t you use animals that are less sentient?

We do test the effects of the drugs on healthspan in non-mammalian system (zebrafish larvae where ageing has been induced by radiation or Drosophila). However, those drugs which show efficacy require testing in mammalian systems for obtaining regulatory approval to proceed testing in patients.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are in contact with laboratories which are the best in ageing research using mice and we continuously exchange tips for best practise. We will monitor the adverse effects very closely, give pain killers to the animals if required or the humane killing of the animal if the adverse effects are likely to exceed moderate severity.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the protocol we have standardised with other laboratories for drug testing on healthspan (Nat Protoc 15, 540–574 (2020)). I keep up to date with the NC3Rs website, twitter and bulletins for any new protocol emerging, which may be relevant to us. I work closely with collaborators worldwide to exchange best practise.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive regular bulletin from NC3Rs which are a useful way of staying informed. My research group and I have attended their regional conferences. We have a track record in contributing towards the 3Rs. I led an initiative called ShARM (Shared Ageing Research Models) to bank surplus tissues from experiments using murine models and donate those to other investigators so that the number of animals bred could be reduced. I have contributed to a piece of work led by NC3Rs on the use of age- appropriate animals in research (Jackson et al. 2016 https://doi.org/10.1177/0023677216653984) to ensure results are more relevant to human conditions. I have initiated the standardization of protocols across laboratories worldwide expert in healthspan testing to ensure we all use the least invasive techniques according to best practise and results are reproducible across laboratories. I support the research of Enrico Dall'Ara on the development of computational models to reduce and eventually replace animal use.

152. Generation, Rederivation and Cryopreservation of Transgenic Animal Models

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

transgenic animal, cryopreservation, rederivation

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged
Rats	adult, embryo, neonate, juvenile, pregnant, aged
Acomys	juvenile, adult, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This service licence will support researchers mostly at our place of work who are working with mice, rats and Acomys (spiny mice). Sometimes, we may also offer our services to researchers at other UK institutions. A team of dedicated transgenic technicians will work under this licence. We generate custom-made animal models with changes in their DNA, or get back existing animal models from frozen material.

Once an animal model has been made, we can freeze it down for safekeeping and sending it to other organisations. We can also help researchers if their animal models are in danger of dying out due to breeding problems. We can help if certain pathogens need to be removed from an existing animal model or building. We will ensure that the researchers who use our services are allowed to do so.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This service licence will give researchers easy access to transgenic techniques. As we can generate models for all researchers at our institution, they don't need licences which cover transgenic production. As we are experts in this field, we can use the most refined and up-to-date techniques. We can obtain animal models from all over the world without bringing disease into our animal units at the same time. By freezing new animal models we protect them from possible disasters and diseases. It is also easier and more refined to share animal models as frozen material than sending live rats or mice.

What outputs do you think you will see at the end of this project?

New genetically altered models for research into disease and normal biological processes.

Who or what will benefit from these outputs, and how?

All the researchers in the biomedical sciences at our institution could benefit from our outputs. Our service will facilitate and optimise access to animal models in the short term for established research groups, and may also allow groups in the future to extend their research into clinically-relevant models through our turn-key services.

How will you look to maximise the outputs of this work?

Internal publicity for our transgenic production services. Presentation at our annual 3Rs symposium on the latest refinements. Pro-active recruitment of all GA strains created or imported to the fully-funded cryopreservation programme. Technical support for adopting refined and novel techniques will be sought from the close-knit community of transgenic core facilities, either by personal contact or through the International Society for Transgenic Technology mailing list and symposium series, which we attend regularly.

Species and numbers of animals expected to be used

- Mice: 52800
- Rats: 5450
- Other rodents: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will be using both rats and mice over the 5 year period of the licence. The scientists requesting our service will determine which species is most suitable for their research. Rodents are the most widely studied and relevant model organisms for the vast majority of biomedical research. The alteration of the genome involves manipulation of foetal life stages, and juvenile and adult life stages are used in the reproductive biology aspects of these protocols. In this respect there are no alternative life stages available for us to work on.

Typically, what will be done to an animal used in your project?

The animals we use to collect embryos will get an injection into their belly. This may involve momentary pain and has an expected mild severity. It may be necessary to repeat this treatment up to two more times. This is better than importing a second group of animals from outside the UK due to the stress of transport over long distances. These animals will be humanely killed.

In order for genetically-altered embryos to develop into live animals they have to be transplanted into "pseudopregnant" females. We perform one surgery on some of the male mice and rats to make them sterile. This has an expected moderate severity even though pain will be managed whilst they recover from surgery. These animals will be used for mating. Mating those sterile mice to females will make the females pseudopregnant and receptive for transferred embryos. We will implant embryos into pseudopregnant female mice and rats using surgery (usually). They will then give birth to those pups. The expected severity for those females is moderate. Once the pups have been weaned, the females will be humanely killed. Animals receiving embryos will only have surgery once. Male sterile animals will be humanely killed at the end of their useful lifetime.

What are the expected impacts and/or adverse effects for the animals during your project?

The surgical processes we will use cause short-term impacts only (a few days), which are minimised with pain relief medication.

We make mice and rats with changes in their DNA for a lot of different researchers. For every animal model we make, we will ask the researcher to predict how the changes in the DNA will affect the animal. We will look out for those predicted changes, but also for anything else which doesn’t look quite normal. Most of the mice and rats we make with changes in their DNA will have no or only very mild adverse effects. For example, some may be smaller but otherwise normal. Others may be sterile. Sometimes the changes in the DNA can make the animals unwell, and this may be of moderate severity. Some animals may develop seizures. Others may have problems with normal breathing. All animals will be regularly looked at by technicians with a lot of experience in working with mice and rats. We use a scoring protocol with defined end points. If any animal seems to be unwell, we will also speak to our vets. Usually, we transfer those animals to the

researchers onto their licences which are reviewed for welfare concerns and humane end-points.

We will give drugs to some animals to switch DNA pieces on or off. The drugs can be given in food or water, or with an injection. The drugs themselves rarely cause adverse effects, and injections are of mild severity. Sometimes the effect of a piece of DNA getting switched on or off can be of moderate severity, as described above. We will use the same scoring protocol as described above. At the end, animals will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For superovulation, all animals will experience mild discomfort. For delivery of nucleic acids in the oviduct, embryo transfers and vasectomy, all animals will experience moderate effects for short periods after surgery only. For animals that are given drugs to induce gene expression or label cells there will be momentary discomfort from the administration process, and no longer term effects.

GA animals that model a disease process that are bred on this licence may have impacts that develop over time, but in general these effects will be managed specifically according to the checks and balances in place in the licences of other researchers.

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Some of the questions our researchers have can’t be answered without the use of transgenic animals. Transgenic animals can’t be made without using other animals. We will only make animal models for researchers who have their own licence which explains why they need to use animals. Spiny mice are a valuable resource for studying wound healing, tissue regeneration and scar formation. This group of animals need to be bred in the laboratory for supply to other projects. This is better than capturing animals from the wild.

Which non-animal alternatives did you consider for use in this project?

Our researchers have looked into alternatives to using animals, and the options vary depending on the nature of the research question. As a community, we are very active in developing cell and tissue culture methods, including growing in dishes small organoids that resemble complex structures in animals. Our institute also has expertise in computational methods and analysis that can model living processes instead of studying them in animals.

Why were they not suitable?

Some of the very complicated biological processes we study (for example, cancer, learning and memory, the development of the body) cannot be studied in a dish, or modelled on a computer. It is the interactions between all cell types in a whole organism and it's environment that are needed to understand the underlying processes and to develop treatments for when these go wrong.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These estimates are based on our accumulated experience over the past 10 years of providing these services to our researchers. Historical numbers have been adjusted based on what we know is likely to happen to the need for our services, and the evolution of better reagents and techniques that reduce the number of animals needed to achieve our outcomes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We do our work using the lowest number of animals possible to make enough animals for the researchers to do their job. We use good protocols and good-quality reagents. We service our equipment on a regular basis. And we record all our data and check if we can further reduce the number of animals we use. We review the literature so we stay up-to-date with any new techniques.

We freeze animal models, so animals don’t need to be bred if they are not used. If an animal model is needed again later, we can bring the animal model back again from frozen material.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Optimisation of numbers used will be achieved in several ways. Control experiments may produce embryos that are not GA. These will be frozen down, and used in future control experiments and to monitor viability of frozen materials over time. Imported animals that fail to mate at the scheduled time can be re-used rather than replaced. Several promising techniques are now available that dramatically reduce the number of animal procedures required to make research models. We are actively establishing these techniques in our group.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The researchers normally decide on the species most suitable for their work in their licence. Most of our customers use mice, but rats are often better for researchers working on behaviour and some human diseases. The most efficient methods for creating animal models are used, predominantly CRISPR/Cas9. For more complex manipulations, it is sometimes possible to achieve the DNA changes in stem cells in the lab before generating a modified strain of animals. This minimises the number of animals required. We will develop expertise in manipulating genes in fertilised embryos without removing them from the body (mouse), but this is technically demanding and will take time to perfect.

This method would use the fewest animals.

Why can’t you use animals that are less sentient?

This licence application focusses on rodents. Other less sentient species are used widely in our institute, where appropriate. Rodents are the best species to study for many areas of biomedical research, and these cannot be studied in as much detail in other species. Body systems such as cardiovascular disease, brain function, learning and memory, stem cell therapies and regeneration of damaged organs, reproductive disorders and others all require mammalian model species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All of our staff are very well trained so when they do procedures like injections or surgery they cause as little pain and distress as possible. Our surgeries are carried out very cleanly, so that the animals don't get ill from the surgeries. All our animals are handled using tunnels or cupping and not by tail- handling.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For best practise in producing genetically altered animals we will follow the guidance in Zevnik, B., Jerchow, B. & Buch, T. 3R measures in facilities for the production of genetically modified rodents. Lab Anim 51, 162–177 (2022). https://doi.org/10.1038/s41684-022-00978-1.

Our facilities follow FELASA guidelines for the exclusion of rodent pathogens.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The regular outputs of NC3Rs and RSPCA are widely disseminated in our organisation. The transgenic team are members of the International Society for Transgenic Technology and the Institute for Animal Technology and regularly attend meetings with other professionals working in this space. The team leader regularly shares new literature that advances the 3Rs and discusses implementation of new methods with the technical team, our vets and our management.

153. The Neurobiology of Primary Headache Disorders

Project duration

5 years 0 months

Project purpose

(a) Basic research

(b) Translational or applied research with one of the following aims:

(i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

(ii) Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Migraine, Headache, Pain, Neuroscience, Therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The pivotal aim of this project is to determine the basic bodily processes that underlie sensory processing, and how these processes are altered in primary headache disorders (e.g. migraine). The ultimate objective is to translate pre-clinical observations to the clinical domain in order that we can better treat these conditions in patients.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This work is important because primary headache disorders represent a major clinical unmet need with considerable socio-economic burdens because of the limited number, efficacy and side effects of existing therapies. Approximately 50% of adults will experience a primary headache disorder annually, migraine alone accounts for up to 16% of the population-that is over one billion sufferers, globally.

Importantly, many of these patients are not adequately controlled by currently available therapies, while many of those used for prevention are associated with significant and often dose and duration-limiting side effects. There is clearly a major need for novel analgesics and disease modifying interventions, and the effort to generate these will be greatly strengthened by an increased understanding of the underlying mechanisms that cause and drive episodic or persistent head pain and associated symptoms. Both episodic and chronic head pain conditions present as a major challenge because of their complexity and range of biological and neuronal processes that change the way in which we perceive head pain. Our work will examine the underlying circuitry controlling and contributing to head pain and primary headache disorders and thus will increase our understanding of head pain neurobiology, such that targeted pharmacotherapeutic or neuromodulatory approaches may be translated to the clinic. Some studies of primary headaches are possible in humans; however, mechanistic questions require invasive techniques that are not possible or feasible at present in humans and as such we propose the work herein.

What outputs do you think you will see at the end of this project?

We anticipate the following outputs:

1. Identifying novel head pain mediators and analgesic strategies. It is likely that the precise role and involvement of these targets will differ depending on the precise disorder. We therefore require a variety of different models to investigate their function. Beyond new targets we are also working on novel lifestyle and neuromodulatory approaches to reduce the incidence and frequency of primary headache disorders.

2. Defining head pain circuitry in health and disease. We will study how neuronal circuits transmit nociceptive signals that lead to the sensation of pain, and how these circuits interact with diverse neuronal networks to give rise to the diverse multisensory dysfunction observed in primary headaches such as migraine (e.g. aversion to light-photophobia). Fundamental questions regarding the integration of peripheral inputs into central nervous systems underlying disease associated symptoms will be determined.

3. The new information generated will be readily shared via conference presentations, peer-reviewed publications, press releases, social media posts and patient newsletters to maximise the impact of the outputs generated.

Who or what will benefit from these outputs, and how?

1) Immediate use of data and products

This work will provide further knowledge of the normal properties and regulation of head pain and associated sensory systems and how they are affected in primary headache disorders. Experimental results will be analysed and used to determine the direction and design of appropriate and efficient follow-up experiments. Data will also be written up and presented at international meetings and published in peer-reviewed journals for dissemination to the research community at large.

2) Opening of new avenues for research

We believe that the data generated from this project will open up new avenues of research. Research into and improved understanding of normal and pathophysiological pain processing and neuronal network function will lead to the determining of cell signals, neurotransmitters, channels etc. implicated in the production of head pain syndromes. Each novel target identified will open a new avenue of research including replication and validation of hypothesis by numerous means.

3) Practical Applications

We believe that the work done in this project will have a future important practical value in the development of strategies to combat primary headache disorders (such as migraine and cluster headache). Previous studies indicate that the discoveries underlying head pain states in animals are transferable to analogous conditions in humans. In short, our hope is that our work will be translated into treatments for patients.

In total the knowledge that could arise from the proposed research would benefit many people within the wider headache and pain communities as we will significantly advance our understanding of the underlying pathology of pain conditions, particularly the recognition that primary headache disorders are associated with maladaptive multisensory integration in the central nervous system.

4) Socio-Economic

Migraine alone impacts over one billion sufferers globally, results in 1 in 4 neurology outpatient appointments in the UK and costs the EU an estimated €20 billion per year. We believe that the work done in this project will have a positive impact in helping to reduce the global burden of disease as a result of head pain disorders.

How will you look to maximise the outputs of this work?

We have a number of external academic and industrial collaborators already in place (including those nationally in the UK as well as those internationally in Denmark, the Netherlands and USA).

Understanding the neurobiology of head pain is of relevance to many audiences including academic and industrial researchers, clinicians, patients and the general public. Different forms of communication must reflect the varied nature of the beneficiaries and project results will be disseminated accordingly such that the importance of this research is described to different audiences in the most meaningful manner. For example, we will regularly write news blogs for patient awareness groups ensuring direct access to those living with the condition.

Results from this work will be presented at national and international scientific meetings with a focus on basic science and translational research. Data will be published in basic science (pre-clinical and clinical) journals including those with an interdisciplinary focus. We will be targeting pain discovery and management communities. Social media such as departmental and institutional twitter feeds will detail project progress, conference attendance and journal publication ensuring that the outputs of this work will be maximised.

Species and numbers of animals expected to be used

- Mice: 10250
- Rats: 4750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are principally interested in the mechanisms that underlie primary headache disorders. Some studies of sensory phenomena are possible in humans. However, the mechanistic questions that we wish to answer require more invasive techniques that are not possible or feasible at present in humans. In vitro techniques are also not sufficiently advanced (and are not likely to be so for some considerable time) that they can model the integrated actions of the peripheral and central nervous systems. Thus, we will undertake some of our work in animals. The use of rats and mice throughout the project, which requires in some areas modelling of a sensitised state, is critically important. The neuroanatomy/neurophysiology of the rat and mouse is well understood and there is a comparable central nervous system complexity to the human. In rats and mice, we can unequivocally establish which pathways underlie distinct pain processing mechanisms where a cell culture system would not model the necessary aspects of pain perception.

The questions we are asking usually relate to a specific primary headache disorder and we commonly seek to reverse translate clinical symptoms, which we need to model with suitable animal models: for instance, migraine typically involves the trigeminal nerve and its central projections, whereas migraine aura is a predominantly cortical phenomenon. We therefore need to study a range of models to recapitulate the human condition.

Some of these animal models are short onset and short duration (hours) and can therefore be studied acutely in animals. For example, the injection of a chemical agent like nitroglycerin that triggers a delayed migraine in migraine patients intraperitoneally in rats and mice is associated with hypersensitivity to touch, akin to the allodynia observed in patients and can be recorded for up to 120- 240 minutes. Other models, like their human counterparts, develop and change over time and so some of these experiments may last weeks or months. For example, persistent exposure of rats and mice to specific acute anti-migraine drugs for up to 15 days to model the medication overuse headache observed in patients is associated with a prolonged hypersensitivity to touch and bright light which can last for up to 3 weeks. The prolonged time course of some experiments and the fact that one of the most important outcome measures in our work is behavioural assessment of the animal, means that only some work can be done on animals under terminal anaesthesia - the remainder will require the use of recovery protocols.

Our scientific questions largely relate to the study of head pain mechanisms in mature animals. However, in some cases an intervention is necessary at an earlier developmental stage. One example is the targeting of cre-expressing neuronal populations expressing Sox14, which is only expressed during the first month of life. As such, cre-dependent adeno-associated viruses expressing chemogenetic tools need to be injected at an early stage in order to facilitate testing the effect of their modulation in mature animals.

Typically, what will be done to an animal used in your project?

Typically our experimental themes (in terms of what will be done to an animal used in this project) will rely on the following sequence steps following determination that animal work is necessary for the question at hand:

1). Where necessary, surgically prepare animals under terminal anaesthesia to permit the measurement of neuronal/vascular activity in real time, using for example, electrophysiology, cerebrovascular monitoring, calcium or optical imaging.

2). Alternatively, in a small proportion of animals surgical preparation will be followed by a period of recovery (at least 7 days), followed by the testing/recording of surgically prepared awake animals under freely behaving (e.g. EEG) or head-fixed conditions (e.g. implantation of indwelling electrodes/imaging devices fixed to the head and then the animal secured to a head post) on a suitable platform.

3). When necessary to identify/manipulate cell populations: genetically label or manipulate the cell population under study, (e.g. inject AAV9-GCaMP6s virus into a rat to

label specific neurons or breed a transgenic mouse line which is knockout for a putative pain mediator.).

4). Apply a treatment/drug (either in the form of a drug or using neuromodulation) so that the outcomes of the treatment/drug can be tested as per steps 1, 2 above or: via behavioural studies: we have begun to innovate with assays that capture more natural, spontaneous animal behaviour – e.g. using home- cage monitoring. We will undertake a variety of behavioural tests of pain sensitivity. Most use threshold stimuli (e.g. applying light mechanical pressure) and the animal is free to withdraw from the stimulus at any time. One, however, uses surprathreshold stimuli (the capsaicin test).

5). Sacrifice the animal through schedule 1 or perfusion for subsequent anatomical studies or gene expression/protein assays.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals used during this project are expected to suffer a maximum of moderate severity. For those used for breeding and maintenance purposes this is expected to be no more than mild suffering and any animal exceeding this will be humanely killed.

Throughout the different protocols used in this project we have taken all necessary precautions to minimize the impact/adverse effects for the animals. A number of our studies are conducted under terminal non-recovery anesthesia to ensure there is no potential for prolonged suffering. Where this is not possible due to the scientific question, animals may undergo some preparatory surgery which can result in transient pain (1-2 days); however, this will be controlled via anaesthetic and analgesic (pain relief) steps during the recovery period.

Recording sessions (behavioural or via implanted devices) in naïve or previously operated animals, respectively may cause transient stress which is minimized by familiarizing the animals with the testing apparatus prior to the recording session. Where pain-responses are measured, these are conducted to establish pain thresholds, with the animal able to withdraw from the stimulus if it becomes uncomfortable.

To study headache, we use several agents which are known to induce or alleviate headaches in patients. Where they cause headache-like responses, doses are carefully chosen to ensure that suffering is kept to a minimum, additionally headache is often associated with an aversion to bright light, so any animals experiencing headache-like symptoms are maintained under controlled low-level lighting. Other agents that are administered are not expected to cause more than mild effects or discomfort in themselves.

In addition, to explore the impact of the environment on headache, some animals will be exposed to abnormal light-dark cycles to model jet-lag like responses. We do not expect

these to cause more than a transient mild impact as the animals adjust to the new schedule. Other procedures will be carried out that are not expected to result in adverse effects, for example, blood sampling will only result in transient discomfort or stress that the animal quickly recovers from.

Finally, to ensure that the level of suffering is kept to a minimum there are strict limitations on the duration of particular interventions. For example, where animals have undergone preparatory surgery, the majority will be humanely culled by 16 weeks, and up to a maximum of 32 weeks in rare cases.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Protocol 1: Mild severity (5000 mice)

Protocol 2: Non-recovery (1000 mice and 2250 rats)

Protocol 3: Moderate severity (2200 mice and 1500 rats)

Protocol 4: Moderate severity (400 mice and 200 rats)

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Some studies of sensory phenomena are possible in humans however, mechanistic questions require invasive techniques that are not possible or feasible at present in humans and as such we propose the work herein. We are principally interested in head pain mechanisms. In vitro techniques are also not sufficiently advanced (and are not likely to be so for some considerable time) that they can model the integrated actions of the peripheral and central nervous systems. Thus, we will undertake some of our work in animals. The use of rats and mice throughout the project, which requires in some areas modelling of a sensitised state, is critically important. The neuroanatomy/neurophysiology of the rat and mouse is well understood and there is a comparable central nervous system complexity to the human. In rats and mice, we can unequivocally establish which pathways

underlie distinct pain processing mechanisms where a cell culture system would not model the necessary aspects of pain perception.

Which non-animal alternatives did you consider for use in this project?

Where possible we work with clinical colleagues to use appropriate human models and tissues . In conjunction, we will use stored animal tissues from previous research and publicly available sources of data to reduce the number of animals required. Finally, we collaborate with local colleagues to use in silico (database mining) and in vitro (e.g. cell culture models) where appropriate to gain preliminary data that can inform or replace animal studies.

Why were they not suitable?

While non-animal alternatives will be used where appropriate, they cannot replicate the biological complexity of the intact animal. The functionality of the circuitries that we wish to investigate, which rely on peripheral and central nervous system processes as peripheral and central manifestations of head pain impact one another, cannot be investigated in vitro and would be too complex to investigate other than in vivo.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used will vary from procedure to procedure depending on the degree of variability in the experimental measures, but our extensive experience with these models has shown that group sizes of 6–8 (rats) and 8-12 (mice) are generally appropriate. For those procedures involving surgery it is scientifically more rigorous to include sham operated control animals in an experiment. However, for techniques which are well established and for which we know from experience that there is no sham effect we will not include such animals in every experiment, but refer to historical control data. The effect of sham surgery will be reviewed periodically and when a new experimenter is using the technique.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I am familiar with the NC3Rs Experimental Design Assistant having used it for my NC3Rs project grant application which regards the optimisation of female rodents in migraine research. The use of this tool when planning experiments means that the numbers of animals being used in the project is at a minimum.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Reverse translation: Clinical therapeutics will form a key starting point for studies . Our main strategy incorporates drug repurposing or exploring the underlying mechanisms of existing therapies to identify disease relevant targets that show increased efficacy and reduced off-target side effects.

This will be combined with:

(1). In silico approaches, conducting literature searches and investigating publicly available channel/receptor expression databases to identify potential ion channel and receptor systems involved,

(2). Where appropriate, ex-vivo tissue preparations from within this proposal and past research will be used to identify which ion channels/receptor systems are altered in human (where available) and animal tissues to shortlist only the most promising targets for use in animal studies, and

(3). Where appropriate, initial testing will be conducted in cultured neuronal and associated cell populations. Taken together the above approaches will ensure only the most promising and relevant drugs proceed to animal studies.

We will further seek to reduce the number of animals studied by careful experimental design, the adoption of sensitive outcome measures with small variation and the study of only the most relevant time points. Where possible each animal will be used as its own control using a repeated measures design. In all in vivo tests the number of animals in each group will be the minimum required to allow valid statistical analysis. It is possible in some instances to supplement the in vivo work outlined by using in vitro cell systems to analyse, for example, the effect of specific compounds on receptor or ion channel expression and we plan to use such studies where appropriate. However, it is not yet possible to make stable cell lines of sensory neurones, our main cell type of interest, or to mimic the long-term effects of medication overuse and chronic sensitisation that occur in vivo. In these instances, the use of animals is unavoidable. By cryopreserving sperm, we will reduce the number of animals culled in breeding programmes.

To further reduce the numbers of animals used and to refine animal use wherever possible, we constantly refine our models ensuring sources of variance including circadian timing and female oestrous cycle can be identified and controlled for in our experimental design and analysis. Tissue samples not used for a specific study, may be collected and

stored for future use, or shared with other groups to minimise the number of animals culled for tissue harvesting. We have developed a large network of scientific and industrial collaborators that permits access to existing experimental datasets that we have screened to ensure our animal work is optimal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

During this project we will use animal models of head pain disorders including migraine and cluster headache. The methods that we will apply include cell population labelling/manipulation, behavioural assays, imaging and electrophysiology techniques. The extensive use of rodents in biological research has already provided considerable information on nociceptive processes. We will limit the severity of our models, for example, a large proportion are conducted as non-recovery where possible. Where recovery is necessary, we limit the time for which animals are kept following surgery or induction of inflammation as much as possible. For example, our chronic sensitisation models result in no overt phenotype; however, animals can be sensitive to external stimuli like bright light. To mitigate for this, we ensure animals are housed under controlled lighting conditions.

We will also be working on a specific examples of refinement, for example, we are applying for NC3R’s funding to refine the use of female rodents in migraine research, a female prevalent disorder. Identifying the most appropriate oestrous cycle stages to test novel analgesics.

The decision to use rats or mice is based on the specific scientific question. Rats provide a more stable physiological environment to conduct detailed in-vivo electrophysiology. Alternatively, mice allow us to benefit from significant transgenic developments, where we can use cre-specific expression to target specific neuronal networks. In addition, a number of transgenic mice exist that harbor human migraine- relevant mutations, permitting the study of the dysfunctional human gene in animals. The majority of our rats and a proportion of our mice will undergo non-recovery procedures, ensuring no adverse effects and none of our protocols fall under the severe category.

Why can’t you use animals that are less sentient?

Due to the nature of our studies, which aim to identify the mechanisms that underlie head pain states, using animals that are at a less sentient life stage is not possible. Pain can only be properly understood via the integration of knowledge spanning molecular, cellular, systems and psychological levels, hence less sentient species are not suitable. For a proportion of the animals that we use, following behavioural and/or electrophysiological experiments, the study will proceed in the terminally anaesthetised animal as ex vivo tissue is harvested for subsequent immunohistochemical analysis for example.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For the majority of surgical recovery procedures (some exemptions sought within), animals will be provided with suitable post-operative analgesia. For establishment of models, suitable post-operative analgesia will be provided in a manner that does not affect the experimental aims. All will be performed under veterinary guidance. Animals will be regularly monitored for post-operative recovery. In addition, where novel models/agents are being used, these will be carefully monitored to ensure they do not cause any unwanted harm.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will publish all our animal work in accordance with the ARRIVE guidelines and the International Association of Pain (IASP) guidelines for the use of animals in research available at: https://www.iasp- pain.org/resources/guidelines/iasp-guidelines-for-the-use-of-animals-in-research/.

We further adhere to the guiding principles for aseptic surgery (https://www.lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf) and the administration of substances (https://doi.org/10.1258/0023677011911345)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Since I regularly use the NC3Rs Experimental Design Assistant tool and actively seek NC3Rs funding I am a regular visitor to their website and interact with the local NC3Rs regional programme manager to explore for 3R’s advancements relevant to our field. As an associate editor of leading research journals that adhere to the ARRIVE guidelines, I am constantly reviewing advances in 3Rs. This allows me to keep informed about advances in the 3Rs, and implement any advances possible to my projects and the wider research community which I am a part off.

154. Development of Nerve Neuroprosthetic Treatments

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Neuroprosthetic treatments, Nerves, Neural implants

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To test, develop, and validate new treatments using neuroprosthetic devices designed to be implanted into nerves to control body function in disease or dysfunction.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Neuroprosthetic implants consist of implantable devices which connect with the nervous system. By reading / writing electrical information into the nervous system, neuroprosthetic implants can affect the function of the rest of the body. Currently, this technology only has a limited use in human patients, in the form of therapies such as deep brain stimulation or vagus nerve stimulation, which stimulate relatively large areas of the nervous system to treat conditions such as Parkinson's disease and depression. However, recent technological advances now allow us to make much smaller devices, capable of communication with small parts of the nervous system and treat many diseases.

Nerve neuroprosthetic implants are neuroprosthetic implants specifically targeted to the nerves of the body. As nerves connect the brain with most parts of the body (including organs and limbs), devices that are implanted in them have the potential to treat a wide range of diseases. This includes diseases arising from problems in the nerves themselves (e.g. nerve injury, amputations, neuropathic pain) or from the organs which they control (e.g. diabetes, bladder dysfunction). Nerve neuroprosthetic implants can not only offer solutions to specific diseases, but represent a whole new class of treatments - one designed to restore health through the use of electrical communication with the body (electroceuticals), rather than making use of chemical treatments (pharmaceuticals).

What outputs do you think you will see at the end of this project?

By the end of this project we expect to have produced nerve neuroprosthetic implants capable of delivering the following treatments in rodents:

Achieve control of bladder function - controlling emptying, prevent unwanted emptying, monitor bladder fullness. (Aim 3)

Monitor activity in the pancreas and liver, and modulate insulin release and glucose blood levels. (Aim 3).

Ameliorate neuropathic pain (chronic pain, arising from damage to the nervous system). (Aim 4). Partially restore sensation and movement to fully paralysed limbs due to nerve injury. (Aim 2).

These three devices will be shared with the scientific community via publications. The first three will also result in devices ready to be introduced into the next stage of work to develop them for use in humans (larger animal studies, human trials). The last one, given its more ambitious scope, will move onto a new licence for further development, with the aim of producing a device suitable for use in human patients in the future.

We also expect to produce (as part of aim 1) a general nerve neuroprosthetic platform (a "general" type of nerve neuroprosthetic implant) - on which we will base all of our other devices. We will share this platform with other labs aiming to develop nerve neuroprosthetic treatments for other diseases. This will also be shared with the wider community through publications.

Who or what will benefit from these outputs, and how?

The development of a"general" nerve neuroprosthetic platform, that can be applied to any type of nerve (aim 1) will be of immediate benefit to the wider scientific community. This will become available before the end of this project, as it will be developed before we begin work on our other project aims. We aim to make a platform that is simple for others to make while providing high resolution connectivity combined with high biocompatibility, increasing its generability and the ability for its translation. There are many other diseases that nerve neuroprosthetic implants could potentially treat aside from those we work on in this licence. Therefore, providing a simple-to-use platform that allows integration of recording and stimulation on a smaller device, designed to integrate and spatially map to nerves at different locations with different sizes will allow other labs to apply these techniques to study a wider range of diseases, beyond those that we will work on under in this licence.

In the medium term, our organ (bladder, liver, pancreas) modulating nerve neuroprosthetics (aim 3) which we expect to have developed by the end of this project will be of use to patients suffering from improper organ function. This includes dysfunction of the bladder (as seen in spinal cord injury patients, 1.5 million patients worldwide), as well as diabetes patients (422 million patients worldwide). Type 2 diabetes would be the most directly treatable condition (by driving insulin release from the pancreas). However, type 1 (as well as 2) could also be treated with these implants (for example, supressing glucose release from the liver). Our neuropathic pain-treating devices (aim 4) are also expected to be of use to patients suffering from chronic pain (an estimated 7% of the population worldwide). In many of these conditions, patients already have access to some treatment options involving implantable electrical devices. This makes it likely that our devices developed in this licence will be ready to follow the path leading to use in the clinic.

In the long term, our sensorimotor-controlling (restoring sensation and movement) nerve implants (aim

2) will in the future be of use to patients suffering from paralysis and/or sensory loss due to nerve injury. Nerve injuries are frequent, with over 200,000 cases of nerve injury reported annually in both the United States and Europe. Given their ability to control sensation and movement, they could also be adapted for use in a wide range of conditions in which these are impaired. This includes other types of injuries to the nervous system (spinal cord injury - affecting over 1.5 million patients worldwide; traumatic brain injury - with an estimated 69 million new cases worldwide every year), amputations (an estimated 320,000 patients in the UK alone, potentially treated with this technology combined with robotic limb prostheses), severe skin damage, etc. Given their more ambitious aim, and the complexities associated with fully reproducing natural nerve activity (instead of simply modulating it),

we anticipate a timescale of 5-10 years following the end of this project before this technology is ready for use in humans.

How will you look to maximise the outputs of this work?

Our group has an expertise in the design and fabrication of neuroprosthetics, as well as in nerve physiology. However, our expertise in the conditions we aim to treat with these devices is more limited. To address this, we have established a network of collaborations with other groups with expertise in these fields. This includes collaborations with other groups working on fields such as bladder dysfunction in spinal cord injury, or diabetes. We will continue to expand our network of collaborations as we search for new conditions which could be addressed with the treatments we are developing.

Once the projects are running, we will disseminate our results through scientific publications, as well as presentations at conferences and seminars.

Species and numbers of animals expected to be used

- Mice: 525
- Rats: 1450

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Implantable medical devices such as the nerve neuroprosthetic being developed in this licence need to be validated in animal models. In order to show that a device is effective, the model needs to realistically simulate the same condition that the device is meant to treat in patients. This is due to the fact that implants face a wide range of challenges - they need to resist temperatures, movement, and chemical and immune system attacks associated with being inside an organism. They also need to avoid causing damage to the tissue around them. Finally, they need to continue delivering therapy despite these challenges for years after being implanted. This combination of characteristics can only be replicated inside of a body.

Rodents have a peripheral nervous system which is anatomically and structurally very similar to that of humans. They can also be trained to perform skilled behavioural tasks. This allows experimenters to develop therapies that attempt to restore advanced skills such as grasping, which also exist (and can be impaired) in human patients. Rodents are also frequently used in the field of neuroprosthetics, meaning that certain experimental results will be available in published literature, and will not have to be repeated.

Typically, what will be done to an animal used in your project?

Approximately 35% of all animals in this work will be used for non-recovery work (terminal anaesthesia). They will not experience any notable pain or discomfort.

For the remaining 65%: animals will typically be trained in a specific behavioural task for 2 weeks. Such a task may be, for example, reaching and grabbing a sugar pellets. After learning the task, they will then undergo a single surgical procedure during which a neuroprosthetic device will be implanted on one of their nerves. The wires from the implant will be run under the skin to a small port fixed to the animal's head. In a fraction of these animals as part of the surgical procedure the nerve may will also be lesioned or ligated

The animal will be allowed to recover and become accustomed to the implant for 1 week after the procedure. It will then be reintroduced to the behavioural task in which it was trained. Typically, this will involve 3 sessions per week for 4 weeks. During the behavioural task, a wireless port will be plugged onto their head connector, and electrical signals will be recorded from their implanted nerve device. As part of certain behavioural tasks (approximately 50% of animals in behavioural tasks) animals may have to be food restricted. This is a commonly carried out procedure in behavioural tasks, done to motivate them to seek food rewards given during the task. We are commited to minimise the welfare impact of food restriction, applying it only when absolutely necessary, and limiting as much as possible when applied. For example, we will typically restrict access to food only the day when the behavioural task is performed.

In a fraction of the animals that have undergone behavioural training and surgery (approximately 30% of all animals), some additional procedures will be carried out. In the weeks when these animals are performing a behavioural task while having their device implanted, some procedures will be carried out to gather more information about the performance of the implant (e.g. imaging, or blood sampling), or to make the implant more stable in the body (e.g. delivery of substances). These additional procedures will provide little additional discomfort to the animal and may be carried out with only a brief period of anaesthesia or (more commonly) no anaesthesia at all.

Some animals (<10%) will also receive one or more injections as part of certain protocols/steps. Approximately half of these will receive only one injection, with the other half receiving multiple - usually a maximum of one injection per week.

What are the expected impacts and/or adverse effects for the animals during your project?

A significant portion of animals under this licence (approximately 65%) will undergo surgery. Animals will generally be drowsy and show limited movement for approximately the first 24 hours as part of the normal recovery from the anesthetic. Animals will also experience some degree of pain around the operated region over the first few days, which will be treated with pain relieving medication (normally over two days post-surgery).

A portion of animals undergoing surgery (approximately 15% of all animals) will receive a nerve lesion. This lesion will occur on a nerve connecting to one of the limbs (forelimb or hindlimb) and will lead to impaired movement and feeling on the affected limb. The nerve lesions will not cause full limb paralysis, as not all limb nerves are injured in the procedure. Depending on the type of lesion and implant used, animals may recover full movement and feeling over the course of ~4 weeks following injury, whereas in the rest no recovery will be seen (approximately 50% of nerve injuries). In hindlimb nerve lesions animals will typically drag the affected limb and show overall limited mobility over the first few days. In forelimb lesions animals will typically retract (lift) the forelimb while moving, but can continue to bear weight on it as only the paw is affected by the forelimb nerve lesion. Lesions will not cause animals to show any difficulty in feeding and drinking. Animals will quickly adapt within days to move around the cage unaffected by their affected limb. Despite dragging the operated limb during the first few days animals rarely develop sores on it, and the ocurrence of this can be further minimised by providing soft bedding for the animals. Nerve lesions do not lead to additional pain aside from that expected from the surgical procedure, and will be treated with the same analgesia regime.

A separate small subset of animals (approximately 5% of all animals) will undergo a nerve ligation procedure during surgery. This leads to the development over several days of hyperalgesia (enhanced pain sensation) and allodynia (pain sensation in response to non-painful stimuli) on their operated paw. This models neuropathic pain in humans. Depending on the severity of the ligation, in the most severe cases animals may keep their paw retracted and bear their bodyweight on the opposite leg. Animals will nonetheless be able to move around the cage and perform daily activities with no major difficulty. A fraction of these animals may also exhibit nibbling of their nails in the operated limb (which may eventually reach their nail bed), but in our experience this is a rare occurence (<5% of operated animals).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect the majority of animals in this work to fall under either the non-recovery or the moderate severity category. Non-recovery should contain all animals used for terminal anaesthesia work (approximately 35%), for both rats and mice. In these animals, all work will be done under anaesthesia, from which animals will not recover. These animals will therefore not experience any pain or discomfort. Moderate severity should contain all animals used in the remaining protocols, for which surgery is performed (approximately 65%). This surgical procedure will mainly be for the implantation of a nerve neuroprosthetic device. These animals will experience short-term moderate pain as a result of the surgical procedure but are generally expected to recover within a few days of the operation.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Nerves make up the circuitry connecting higher structures of the nervous system (brain, spinal cord) with the rest of the body. As such, they are an ideal structure to target to develop therapeutics for a wide range of structures (bladder, pancreas, muscles, etc.).

An effective nerve neuroprosthetic device must be able to interact with nerves for prolonged periods of time. This must occur despite the numerous challenges that a body poses (immune system, movement of muscles, degradation of implant materials). An effective device must also be able to deliver therapy with no side effects - for example, activating nerves to contract the bladder, but without unintentionally activating other fibers carried by the nerve leading to a sensation of pain.

The wide range of interconnected body systems that play a role in the effective delivery of electroceutical therapy using nerve neuroprosthetics makes the use of animal models essential in their development.

Which non-animal alternatives did you consider for use in this project?

The following systems were considered for use in this project instead of animal work:

Ex vivo (tissue in-a-dish) nerve systems - culture systems in which nerves, harvested from animals, are kept in dishes for periods of days to weeks. We considered testing, developing and validating our nerve neuroprosthetics on these nerve in-a-dish systems.

Computer simulations - simulations incorporating properties of nerve tissue and of materials from which the implants are made. We considered designing and developing our devices based on the information gathered from these simulations.

Why were they not suitable?

While all of the above mentioned alternatives will be used for this project (optimising nerve neuroprosthetic technology before moving it into the animal testing stage), these techniques cannot fully replicate the complexity of living organisms.

Ex vivo nerve culture systems can be used to test the interactions between implants and nerve tissue itself, but present many limitations. Firstly, nerves can only be cultured for a

maximum period of weeks. This is too short of a period to model long-term (months) interactions between tissue and implant.

Secondly, this culture has to be done outside of the body, with no blood circulation and therefore little presence of the immune system (a key player in implant rejection). This further limits the relevance of ex vivo systems in studying long-term interactions. Finally, while good for studying the effect of neuroprosthetics on the nerves themselves, delivery of treatment with neuroprosthetic implants is not limited to a simple interaction with nerves. Nerves need to be activated in specific patterns to produce a desired effect in its target organs/muscles. Alternatively, nerve activity needs to be carefully read and disentangled to understand what particular patterns of nerve activity mean. Ex vivo nerve systems lack both target structures and naturally-occurring nerve activity, and are therefore insufficient to simulate the complexity of a full organism needed for use patients. Further, the immune system is lacking in ex vivo models, resulting in an insufficient mimicry of the actual implantation and subsequent healing process. To adequately observe and design for immune response (foreign body response, immune reaction, etc.) with regard to placement of implants, in vivo studies are necessary.

Computer simulations help in understanding the nerve-tissue interactions. However, they present similar limitations as ex vivo systems in providing an understanding of the effect the implants will have on the entire body. Simulations can be used to predict how implants will activate nerves, or whether nerve activity will be able to be read by an implant under given conditions. However, there is currently not a deep enough understanding of the how natural patterns of activity are distributed throughout nerves, or how activating different portions of a nerve or in different temporal patterns can result in different effects in their target organ. These factors are key in order to ensure that the nerve activity introduced with the implant results in the desired changes in the target organ to treat a condition.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimates of animal numbers are derived from a combination of 2 factors. The first is our experience in developing nerve neuroprosthetic devices - particularly that derived from our previous licence. The second is our engineering knowledge on the typical number of iterations (versions of devices) necessary to develop a successful neuroprosthesis. Over the course of this licence, we expect to develop a progressively improved neuroprosthesis device. From our previous experience, we expect to have to go through approximately 15 iterations of devices before arriving to its final version. This final device will then be applied

to different areas: sensorimotor (nerves controlling movement and sensation), organ modulation (nerves controlling organ function), and neuropathic pain (dysfunctional nerves causing chronic pain) models to develop electroceutical treatments. Each of these will likely require a further 10 iterations to optimise the device for each specific application.

From our experience, each individual iteration of devices/electroceutical treatments requires approximately 25 animals to test and obtain decisive feedback on. While difficult to predict the exact numbers at this stage, this calculated estimation provides a good approximation to the number of animals we expect to use.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During experimental design, we accounted for the large volume of work we will include in each iteration of devices prior to testing them in living animals. This includes engineering tests (testing devices on the bench), computer models, and cell culture tests. This will reduce the number of iterations necessary to reach the "final" device or treatment.

We also made use of PREPARE guidelines and the NC3R experimental design tool, ARRIVE guidelines, power calculations, and knowledge of literature in our field (neuroprosthetic devices) to provide an accurate estimation of the number of animals that will be necessary to test each iteration/ We will continue to use these tools to design our future experiments. The estimation of 25 animals per iteration accounts for pilot studies, non-recovery work, and surgical implantation with behavioural testing to provide conclusive results with the least number of animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

A typical experiment will involve pilot studies with a few animals to explore the efficacy of an implant being tested. If these look promising, a larger number of animals will be implanted with devices.

Numbers for experiments will be estimated based on the initial pilot studies, together with statistical power calculations. This will increase the likelihood that if our device is effective we will be able to observe and measure it in an experiment.

We will also include numerous non-animal tests for our devices prior to moving them on to animal work. This will include in vitro tests, engineering characterisation, and computer simulations of their short- and long-term behaviour when implanted. This will allow us to increase the success rate of our implantation experiments and minimise the number of in vivo experiments required.

In our experiments, data will typically be acquired and processed using automated equipment and software wherever possible, in order to minimise bias.

In almost all cases, animals in our licence will be obtained from commercial suppliers (including immunodeficient lines). This should eliminate any animal waste due to inefficient breeding.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The work in this project will be carried out in mice and rats, with the majority of the work carried out in rats. Mice will be used in cases where further work (not covered by this licence) leading to clinical translation is likely to require certain models found only in mice. For example, mouse models of neurodegenerative diseases which develop motor deficits - these deficits may be addressed using our neuroprosthetic devices and translated to the clinic for treatment of patients suffering from these conditions but will require preclinical validation in mice.

Most experiments will require the implantation of a nerve neuroprosthetic device into the body. In some cases, a nerve injury will also be carried out. This will be done to simulate a human condition we aim to treat with the implants.

Implantation of a nerve device is well tolerated in rodents, when surgery is performed under adequate aseptic conditions and pain relieving medication is provided. The implants are developed and optimised in vitro (i.e. using cells cultured in-a-dish) prior to use in animals. Our devices are also typically made to be small, flexible, and overall compatible with tissue. This makes them unlikely to cause any discomfort to the animal while remaining implanted.

Nerve injuries in this licence will only be carried out on one nerve per animal. This nerve will always be a sensorimotor nerve - a nerve that provides feeling and movement to a limb. This results in a partial loss of movement and lack of sensation in one of the animal's paw. This class of injury is well tolerated by rodents. Within a few hours of recovery from anaesthesia, animals can typically be seen moving around the cage making use of their three limbs and some function preserved in their affected fourth limb. Despite causing little trouble during normal behaviour (movement around the cage, grooming), this injury does impair finer tasks such as pellet grasping. The experimenter can make use of this to test devices aimed to restore similar deficits in patients. The effects on welfare combined with

its use as an injury model make limb nerve injuries an ideal model for the development of this technology.

Why can’t you use animals that are less sentient?

Certain parts of this project can be carried out in terminally anaesthetised animals - including short- term tests of nerve neuroprosthetic devices and testing of electrical parameters. For this reason, protocol 1 of this licence is centred around non-recovery work and makes up a significant portion of the animals used in this licence. However, an effective implantable neuroprosthetic treatment not only has to activate/monitor a structure, but it must also do so throughout prolonged periods of time. This presents challenges due to factors such as animal movement, inflammation, and degradation of materials. All of these factors are expected to occur in human patients, and can only be reproduced in animals where the implant remains in the body for long periods of time.

While less sentient animal species do have nerves in their bodies, the system of nerves found in humans is unique to vertebrates. Rodents, specifically, have an anatomical distribution of nerves very similar to humans. Further, some of these nerves are of similar order of magnitude in size to those of humans, increasing the translatability of the devices. Rodents, and rats in particular, are also frequently used as surgical models, and their anatomy is very well characterised in published literature. Rodents are also able to learn a wide range of behavioural tasks and can be used to test the effectiveness of treatments in situations similar to those potential patients benefitting from these treatments may encounter. These factors make rats an ideal model in which to develop nerve neuroprosthetic devices to develop electroceutical treatments aimed at human use.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be group housed whenever possible. Group housing can be problematic when animals have been implanted with devices for electrical recordings. This is because rats often nibble on the ports which house the wirings of the implants - referred to as the headcaps. We will use strains such as Lewis rats which more rarely exhibit this behaviour. We have also optimised the shape and material of our headcaps as part of our previous work to the point where animals are able to be group housed while wearing them.

These headcaps are also often the main source of discomfort for animals with an implanted neuroprosthesis. Headcaps are designed to protect the wires while providing a point of connection to the device for the experimenter to use. However, they can be bulky, difficulting animal movement around the cage and normal grooming. In order to minimise the impact of our headcaps, we are designing and fabricating our own headcaps using 3D printing. This allows us to make the headcaps as small as possible to fit our devices, and with materials and structures which minimise their weight. Our current headcap designs weigh approximately 12 grams, which is <5% of the bodyweight of rats typically used in

these studies. We will continue to optimise our headcap designs as we carry out our project.

Surgical procedures will be carried out by licence holders competent in the procedure and following LASA guidelines for aseptic surgery. Preoperative and postoperative care will be provided to animals by trained animal technician staff. Following surgical procedures, all animals will be provided with wet mash food or gel to support them during postoperative recovery. Analgesia will be used post-surgery on all cases (typically over two days post-surgery).

Behavioural tasks often requires animals to be food restricted to provide an incentive for animals to learn a particular task. We will work to minimise the amount of time during which animals have to be food restricted. We will only limit access to food only to the day of training (fasting). Fasting will typically be done three days (maximum five days) per week, allowing for one day of free access to food between each session.

Supervisors teaching new PILs will do so following LASA guiding principles to ensure new users are properly trained to perform any procedures necessary.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All users of this licence will be required to read the published ARRIVE and PREPARE guidelines prior to start of any work and integrate these into their plan of work.

Supervisors teaching new licence holders will do so following LASA guiding principles to ensure new users are properly trained to perform any procedures necessary. This includes developing and making use of training protocols for each procedure. All work will generally be carried out in the same facility, allowing new users to have routine contact with supervisors to ensure good transfer of knowledge.

All surgical work will be carried out following LASA guiding principles on preparing and undertaking aseptic surgery. LASA guiding principles for behavioural laboratory animal science will be followed when undertaking behavioural work.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Individuals involved in this project will remain updated on advances in the 3Rs through a variety of sources, such as: participation in animal facility user meetings, subscription and participation in relevant mailing lists and workshops in our institution, routine browsing of the NC3R website and of relevant published literature. We will also hold periodic meetings to review the current state of all work under this licence and coordinate the implementation of any improvements to our work based on the 3Rs.

155. Effect of Wheat Bran and Probiotic Intake on the Oxidative Status in the Colon and Blood of Finisher Pigs

Project duration

2 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Pigs, Wheat-bran, Probiotics, Pig health, Gut health

Animal types	Life stages
Pigs	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this research is to understand the benefits of feeding wheat-bran alongside a probiotic on markers of health such as oxidative stress and inflammation within the gastrointestinal tract.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Wheat-bran contains Ferulic acid that has previously been shown to have beneficial effects on pig and human gastrointestinal tract health. However, its bio-accessibility within cereals is limited, reducing the capacity for beneficial effects. By combining wheat-bran with a probiotic, such as Pediococcus acidilactici or Lactobacillus plantarum, it is hypothesised that ferulic acid could become more accessible to the pig. Such probiotics result in the degradation of cell wall polysaccharides which may release bound ferulic acid, and therefore promote benefits such as reduced oxidative stress and inflammation.

What outputs do you think you will see at the end of this project?

The results of this research could be beneficial in determining whether the inclusion of a probiotic alongside wheat-bran could increase the bioavailability of ferulic acid and promote gastrointestinal tract health in finisher pigs. If significant improvements are observed, modification of pig diets to include wheat bran and a probiotic could improve colonic health through promoting the growth of probiotic bacteria strains within the colon, increasing short chain-fatty acid concentration, reducing the number of pathogenic E.coli species present as well as reducing inflammation through an increased release of ferulic acid. Furthermore, the results could lead to further work to increase the use of wheat-bran instead of wheat in pig diets as means to also reduce feed costs. By gaining a better understanding of the availability of ferulic acid with a probiotic, this could support collaboration with human nutrition and health researchers to improve human gastrointestinal tract health, given the similarity between the pig and human gut. This could be beneficial for understanding how to reduce human diseases associated with oxidative stress, such as diabetes, cancer and cardiovascular disease.

Who or what will benefit from these outputs, and how?

The benefit of the outputs will not be realised until all laboratory analyses are conducted after the trial has been complete. Once outputs have been assessed, data and information will be published.

Results obtained from this trial will be beneficial for future researchers in the area to show whether beneficial effects are observed. This could lead to subsequent performance trials to determine its validity for use in pig feed, which could lead to reduced feed costs for farmers. In terms of human application, this work could provide human food and health researchers with an avenue for further investigation of wheat bran and probiotic consumption for potential application to reduce oxidative stress related diseases specifically in the colon.

How will you look to maximise the outputs of this work?

The results obtained from this study will be published regardless of whether they are the expected outcome. This work will be in collaboration with animal and food scientists and therefore outputs will be shared across the pig industry as well as across researchers in animal and human health and nutrition.

Species and numbers of animals expected to be used

- Pigs: 36 pigs

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The species chosen allows for results to be applicable to commercially farmed pigs alongside humans. Finisher pigs have been selected because they have a more stable gut microbiome and are less immunologically challenged compared to younger pigs, such as those at weaning. Determining the effect of these diets in a stable gut microbiome can enable more accurate results from the gastrointestinal tract microbiome and can increase consistency of the results compared to the use of younger pigs, whose gut microbiome can be highly varied.

Typically, what will be done to an animal used in your project?

Thirty-six pigs will be selected to go onto the trial at approximately 16 weeks old, they will be fed one of three diets (Control diet; control diet + 20% wheat bran; control diet + 20% wheat bran + probiotic) until approximately 22 weeks of age. During this time frame, all pigs will have blood samples taken on day 0 (16 weeks old) as well as days 14, 24, 34 and 42 of the trial. Ten pigs per treatment will be randomly selected to be humanely euthanized under Schedule 1 of the Animals (Scientific Procedures) Act 1986, the remaining six pigs will return to the commercial herd for commercial slaughter. Ten pigs per treatment will allow suitable power for significant differences to be observed with regards to gastrointestinal tract analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Blood sampling: Possible adverse effects include short-term discomfort whilst being restrained as well as at the injection site during and immediately after collection of blood. However, home office trained technicians will complete the sampling and therefore will ensure the animals comfort and minimise stress. Samples will be collected using aseptic techniques. All pigs will be monitored to ensure infection of the injection site does not occur, by carrying out daily health checks.

Euthanasia: At week 22 ten pigs per treatment will be randomly selected to be humanely euthanized, the remaining six pigs will return to the commercial herd for commercial slaughter. Animals will be killed humanely and appropriately based on methods described in the Animals (Scientific Procedures) Act 1986, Schedule 1. Based on this a captive bolt will be used, followed by exsanguination to confirm death and prevent prolonged pain and/or distress.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild severity for all pigs (36) within the trial.

What will happen to animals at the end of this project?

- Killed
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The program of work aims to identify whether the use of a probiotic in conjunction with a diet containing 20% wheat-bran enables more ferulic acid to be available within the gastrointestinal tract of pigs and humans. It is not feasible to conduct this form of work in humans given the requirement for collection of colonic samples at the end of the trial to determine effects on the microbiome and therefore pigs are the most appropriate animal to use. Furthermore, given the benefit of these experimental diets could be applicable to commercial farming, it is essential to determine their effects on commercial housed and reared pigs.

Which non-animal alternatives did you consider for use in this project?

In vitro work was considered to complete this work.

Why were they not suitable?

As there can be a dynamic interaction between the host, and their gastrointestinal tract microbiome, in particular the colon in the case of ferulic acid, it is not feasible to replicate this work in vitro.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

A sample size has been determined using information from similar studies that have found differences in markers of inflammation and oxidative stress in response to probiotics in pig diets (Yin et al., 2004; Molist et al., 2010).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A power analysis has been conducted in R (v. 4.1.3) to determine the sample size, using a One Way Anova. This calculation included an effect size of 0.6, a significance level of 0.05 and an 80% probability of results not being due to change. This resulted in a sample size of 9.9906. To allow for production losses, 12 pigs per treatment will be used to ensure significant differences could still be obtained if pigs are removed from the trial or cannot be sampled.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The same animals will be used for sampling at each time point to reduce the total number of animals sampled and to enable accurate results to be determined over time. Ten pigs per treatment will then be humanely euthanized at the end of the trial to collect further samples for identification of microbial differences within the GIT, minimising the likelihood of further investigation.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The trial will run under commercial pig farming conditions. To measure oxidative stress and inflammatory markers over time in pigs, blood samples are the most appropriate method to use. The use of pigs allow potential beneficial effects to be applicable for

commercial pigs as well as humans. Blood sampling will be conducted by fully trained and licenced personnel and therefore pain and suffering will be minimised.

Why can’t you use animals that are less sentient?

Given there can be interactions between the gastrointestinal tract microbiota and the host, and that the products used in this research could be beneficial for commercially raised pigs, it would not be appropriate to use any other species than commercially reared pigs.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The regulated procedures involved will not cause pain, suffering or lasting harm more than mild severity as these procedures will be carried out by fully trained staff that possess a Home Office Personal Licence. If blood cannot be taken after 2 attempts the animal will be returned to the pen without a sample collection. During the entire research trial, all pigs will be health checked daily to ensure the health of the pigs is maintained. Any pigs showing signs of ill-health, as determined by trained research technicians and/or the veterinarian, will be treated with relevant medication or euthanized appropriately. A dedicated pig veterinary specialist will be available in these situations.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3Rs guidance on blood sampling pigs from the external jugular vein (non-surgical). Available at: https://www.nc3rs.org.uk/3rs-resources/blood-sampling/blood-sampling-pig#anchor_4.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Regularly check the NC3Rs website and read the regular emails received as part of the licensee email list from NC3Rs as well as actively look for advances in the area that could effectively advance the research.

156. Mechanisms and Benefits of Sleep and Links With Sedation

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Sleep, Sedation, Insomnia, Brain, Aging

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We will address several questions centred around sleep’s restorative properties:

A, what types of brain cells sense the need to sleep when we are sleep deprived, and can sedative drugs artificially promote these natural restorative sleep pathways?

B, whether sleep promotes the removal of waste products from the brain? C, does insomnia or sleep loss speed the onset of dementia?

D. does sleep quality decline with age?

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We sleep for 30% of our lives, but why we do remains a mystery. Why do things feel better after a good night's sleep? We will use the latest genetic, physiological and behavioural techniques with mice to identify the brain wiring involved. Our work will aid understanding of processes that sense the need to sleep, and identify whether drugs can be developed that provide natural sleep's restorative benefits; we will determine whether side-effects of sedative drugs can be reduced. Our work could allow new approaches to improve mental health by having better sleep. Furthermore, there is speculation that sleep disturbances in healthy people, repeated over years, could accelerate biochemical processes leading to dementia. Mechanisms responsible for how disrupted sleep might, in the long-term with ageing, increase the risk of getting dementia are unknown. Does sleep promote removal of waste metabolites from the brain, for example, and does removal of such toxic waste chemicals decrease with age or with the onset of dementia?

What outputs do you think you will see at the end of this project?

First, new information; we have a strong track record for basic research into mechanisms of sleep and sedation. Our research could answer the question of why we need to spend a third of our lives sleeping, and will lead to conference presentations and peer reviewed publications in open access journals. Second, we anticipate that our research will identify new treatment options for better sedatives and drugs that improve the quality of sleep.

Who or what will benefit from these outputs, and how?

In the short and medium term, scientists and stakeholders in industry will benefit from our research results in deepening our understanding of this most mysterious brain state, sleep. In the long term, our work will aid understanding of feedback processes that sense the need to sleep, and identify whether drugs can be developed that provide natural sleep's restorative benefits; we will determine whether side-effects (e.g., low body temperature) of sedative drugs can be reduced. Finally, our discovery of sleep-promoting circuitry could allow new approaches to improve mental health. Also, in the long-term, based on our work, new treatments for dementia could be devised that are non-pharmacological and non-invasive, for example, by enhancing sleep. In the long-term, a greater understanding of how sleep might clear toxins from the brain may allow the development of interventions that harness or strengthen these natural processes.

We want to understand why we sleep and if agents can be developed which give the benefits of sleep.

Scientific benefits: How (and why) we spend about 30% of our lives in a state of vulnerable inactivity, sleep, is an enduring mystery. Like hunger, thirst and sex, the urge to sleep is one of the fundamental and primal biological drives, and with ageing, sleep drive tends to decline. The sleep drive builds during waking in proportion to the amount of time spent awake and then dissipates as we sleep. But what exactly the sleep drive is, as

reflected in the increased need to sleep after sleep deprivation, remains unsolved. It is also unknown from where in the brain the sleep drive originates. And intriguingly, how and why this sleep drive is affected by age remains unknown. Understanding the circuitry involved in sensing and generating the drive over time is an essential prerequisite to later tackling the most baffling question of all – what physiological need is satisfied by sleep?

Medical benefits: Patients in intensive care often have to be maintained in sedated states for prolonged periods, and people undergoing day surgery or investigative procedures must be sedated and immobile, yet remain arousable and compliant. But anaesthetics are not benign - they can cause cognitive deficits in the old and can be toxic in neonates. Understanding how sedatives work could lead to drugs with fewer side effects and which might give the restorative benefits of natural sleep.

Lack of sleep undermines health: large and abnormal changes in biochemical metabolites occur in otherwise healthy people following lack of sleep or mistimed sleep. The prevalence of sleep disorders, and the number of people prescribed sedatives is growing. This results in an enormous societal and economic burden. Disrupted patterns of sleep due to shift work are increasing, as are the multiple factors that disrupt sleep in our 24-hour society. Inadequate sleep, for example, is a major cause of traffic accidents.

How will you look to maximise the outputs of this work?

We disseminate new knowledge through interaction with academics at conferences and by publications in peer-reviewed journals, and by engagements with the public through outreach events or by giving interviews with the media. We publish all results regardless of if they prove, or disprove, our hypotheses.

Species and numbers of animals expected to be used

- Mice: Over 5 years we anticipate to use approximately 7000 mice.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All of the work in our lab is with adult mice. The power of the molecular genetics approaches we plan to use in mice, together with the ancient evolutionary origin of sleep, give us confidence that our results on core physiology will be generalisable to humans. Mice and humans have the same sleep structure and regulation, the difference being that in humans, sleep is consolidated into a 7- to 8-hour block, whereas mice have distributed naps throughout the 24 hours. Although the timings of sleep do differ in mammals, which could be related to brain size, ecological lifestyle and circadian (time-of-day) factors, we believe that sleep in mammals has a core physiological purpose (e.g., toxin clearance),

and this core evolutionary function will be conserved. Another strong reason to use mice is that a wealth of data on mouse brains is now available. The behavioural tests to be applied are experimentally robust and mice can perform them readily. Most importantly, mice have readily modifiable genomes. This has resulted in many useful mouse lines that we can use.

Typically, what will be done to an animal used in your project?

Our work involves the introduction of gene agents (viral vectors) by surgery, using a computer-guided injection system that allows accurate positioning of the viral vector inside the brain. These gene agents usually make specialised proteins that can sense light (a technique known as optogenetics) or that can uniquely sense particular chemicals (one of these chemicals is called clozapine-N-oxide, an otherwise inert chemical, and the technique is known as chemogenetics). The principle is that only particular types of brain cell become sensitive to light in optogenetics or the chemical clozapine-N- oxide in chemogenetics. The introduction of gene agents is often accompanied by the subsequent placement of electroencephalogram (EEG) electrodes (to measure brain activity) on the connective tissue layer that covers the brain, together with an electromyogram (EMG) electrode inserted into the neck muscles to measure muscle activity, and/or additional cannula implantation, or the insertion of a telemetry device into the abdomen to record core body temperature. After achieving full recovery from surgery, various behaviours of animals (e.g., sleep/wake, sedation, exploration in an open area) are typically monitored. We often examine how drugs such as sedatives and anaesthetics affect the electrical activity patterns (EEG) in the brain, and from which types of brain cells (neurons) these activity patterns originate. We also use light given to specific parts of the brain via an optic cable to selectively switch on or off particular types of brain cells and see how this affects the ability of the animal to respond to sedative drugs or if these neurons help control the animal being asleep or awake. Alternatively, in some cases, sleep-promoting and sedative drugs are given via intra-peritoneal injection or through a cannula. We also do simple and gentle sleep deprivation experiments with the mice, whereby mice are given simple novel objects, such as Lego bricks; usually mice are always taking naps, but exposure to novel objects keeps them awake; we then let the mice sleep and again monitor their brain waves (EEG). We also look at how environmental temperature influences the ability of mice to sleep; for example, we study how placing the mice at 32 degrees C (a comfortable temperature for mice) influences their tendency to sleep. We may also look at how mice perform in certain simple behaviours, such as memory tasks, such as a choice of novel objects or if they prefer to be in dark or light places. Animals tend to be single housed. A minority of animals are aged up to 18 months so that the effects of sleep on ageing (and vice versa) can be monitored. After the experiments, animals are humanely killed by either schedule 1 or transcardial perfusion fixation under terminal anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Surgery has the main impact on the animals. Surgical procedures are expected to cause some moderated discomfort that animals will fully recover from quickly. They will be given pain killers and post-procedural treatments and checks just like people are. In general, the expression of foreign genes in specific brain regions does not cause any adverse effects in our mouse models. Other procedures, such as administration of substances, are expected to cause minimal but transient discomfort with no lasting harm. The drugs administered may cause sedation/anaesthesia, or sometimes wakefulness. During sleep deprivation, mice are exposed to novel objects (such as Lego bricks) or a few hours; this tends to keep them awake without any lasting harm, the same as if people stay up for a few extra hours in the evening. Selected parts of the brains of conscious mice are sometimes stimulated with light (optogenetics); the mice are unlikely to be aware of the light stimulation, but may become transiently sleepy or more awake. Similarly, for the chemogenetic technique, whereby we give a harmless drug such as clozapine-N-oxide to the mice, this may make the mice transiently more sleepy or more awake, but again this is unlikely to cause lasting harm. We may sometimes place mice for a few hours in warmer or cooler external environments and examine how the mice sleep; mice live naturally in a wide range of temperatures, and we will mainly use temperatures such as 32 degrees, which is one of the most comfortable ambient temperatures for mice. The simple behavioural tasks that we give mice to test their memory or preference for dark or light places are unlikely to cause lasting harm or distress. In many of our experiments, however, mice will be single housed, sometimes for the duration of their lives. This single housing could increase their stress levels, but they will be provided with cage enrichments. We will also be looking at sleep in older mice, which requires us to keep them up to 18 months of age; ageing is a natural process, and some mice may well physically suffer simply by becoming older, just as is the case for humans; on the other hand, although mice in the wild are unlikely to live that long, 18 months is not a truly old age, and might be roughly equivalent to humans in their seventies; in other words, we are not undertaking extreme ageing of the mice, and any discomfort they may experience from ageing will be proportionate. Finally, during procedures under general anaesthesia without recovery, the depth of anaesthesia we give to the mice will be sufficiently deep that the animal will feel no pain. We do not anticipate any severe adverse events.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- 100% moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To understand the brain wiring (neuronal circuitry) underlying sleep, sedation and how this wiring changes with ageing, the use of animals to study integrated behaviour is essential. Cell lines or computer simulations cannot yet come close to reproducing the brain's complexity. Although the properties of isolated neurons can be studied ex vivo, and even in brain organoids, linking these neuronal properties with animal behaviour absolutely requires intact animals. We use ex vivo and in vitro approaches to optimize molecular methods before undertaking experiments in intact animals; for example, we have spent a year optimising a new method to measure metabolite clearance using jellies containing milk as "phantom brains", to mimic opacity and density of the brain (grey matter), before undertaking in vivo experiments. In lay terms, the method we developed in the jelly involves filling the jelly with a dye, and permanently bleaching a circular region of the dye in the jelly with a light beam, watching under a microscope the dye diffuse back into the bleached region, and then discovering a mathematical equation to determine how fast the dye from the unbleached region moves back into the bleached circular region. Having demonstrated that this worked in the jelly, we then changed the maths slightly to allow us to use this method in a living brain (in the jelly, it was a circle of dye; in the living brain, it is a sphere of dye – but the principle of the method, bleaching a dye, is the same).

All the artificial genes we use to express proteins in the brain our tested in cell lines in our lab before we use them in vivo.

Which non-animal alternatives did you consider for use in this project?

There are currently no scientifically validated alternatives available. Note, however, we have spent a year optimising a new method to measure metabolite clearance using jellies containing milk as "phantom brains", to mimic opacity and density of the brain, before in vivo experiments.

Why were they not suitable?

Not applicable.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of mice is based on the licence covering 5 years and the number of project aims covered under the licence.The numbers are also based on our previous experience generated over the last 15 years of work on sleep-wake behaviour as part of previous project licenses.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The lead scientist first obtained a PPL in the early 1990s. We have over 30 years experience designing mouse experiments and always aim to minimise numbers as much as possible through:

randomisation- validation of endpoints and biomarkers in pilot experiments to generate baseline data for subsequent power calculations. careful selection of endpoints and biomarkers to reduce variability and hence n numbers- use of the NC3R EDA tool.

ensuring that experimenters are trained to high standards.

careful planning and discussion of experiments to ensure all appropriate control groups are included in the design.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Each week we have a users meeting in the lab, where we discuss all ongoing mouse experiments and where the current mouse numbers under our PPL jurisdiction are circulated.

We also:

Conduct pilot studies to generate data for subsequent power calculations.

Use a histology core facility to ensure optimal processing, cutting and staining of brain tissue sections.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use adult genetically modified mice imported to our licence from commercial breeding facilities. We manipulate and monitor targeted parts of the brain wiring (neuronal circuitries) of the mice by precise computer-guided surgeries (for example, injecting genes to make particular types of brain cell uniquely sensitive to light, a technique known as optogenetics) and implanting devices that measure brain waves (known as the EEG). The EEG signal enables us to precisely determine the type and stage of sleep or sedation a mouse is in. We then test different types of drugs to examine their effect on sleep and can, for example, use light pulses to artificially activate small parts of the brain to examine if this induces sleep. By using these types of method, we can obtain precise behavioural results to answer our research questions. We also use untethered recording devices that minimises the risk of causing unwanted suffering or distress to the animals. Operators for each technique are trained with designated trainers in our team and their technical competencies are assessed by the Named Veterinary Surgeons and Named Training and Competency Officers.

Why can’t you use animals that are less sentient?

Less sentient animals are further apart from human biology. It is critical to perform these studies in mammals since there are significant differences between the physiological systems of frogs and fish to humans. The mouse is the lowest mammalian species in which the full range of genetic and physiological manipulations necessary for the investigation of sleep and wakefulness can be achieved. We cannot use animals that have been terminally anaesthetized because the basis of studying sleep- wake behaviour is that animals are awake and then asleep, or can be sedated from an awake state.

Thus, in order to understand the regulation of these behavioural states, we do need animals to be awake before they go to sleep or become sedated.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

If required for some experiments, we may increase post-treatment monitoring and post-treatment care.

Refinements to assess sleep and circadian changes in mice: we have redesigned an open-source wireless device to uninterruptedly track the movement of mice with minimal investigator intervention. We have also optimised a non-invasive method to continuously monitor the levels of stress of the animals, by measuring levels of the stress hormone corticosterone in the droppings. These refined experimental tools allow the consistent analysis of sleep-associated symptoms in the animal models used in our laboratory.

We use a surgical frame with an integrated brain atlas to assist with surgery. Software adjusts the coordinates of injections and electrode placements, improving accuracy and reducing the number of animals that need to be used. This set up also allows us to store target coordinates and calculate positions relative to the injection point on the skull measured for each animal, reducing surgery time and speeding up recovery.

In regular meetings, we also share experiences in animal handling amongst the team to ensure everybody uses the most advanced methods.

Procedures are carried out by experienced PIL holders and overseen by NACWOs who are familiar with the procedures we use. Surgeons for each surgical technique are trained by designated trainers in our team and their technical competencies are assessed by the NVS.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are several best practise guidelines that are published and that we will adhere to: ARRIVE guidelines, LASA guidelines, PREPARE guidelines, NC3Rs guidance, as well as locally published guidelines from our internal committees.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will carefully review all information communicated by the Home Office and establishment staff/relevant named persons as well as our establishments 3Rs group. In addition, we will keep updated with the NC3Rs website and relevant other activities.

157. Deciphering Neural Circuits that Integrate Visual and Somatosensory Cues for Avian Flight

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

sensory neurobiology, multisensory integration, bird, neuroanatomy, locomotion

Animal types	Life stages
Taeniopygia guttata	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Flying birds integrate sensory information to support diverse strategies for manoeuvrability and stabilisation that do not rely on feedback from the ground. The brainstem and cerebellum are key brain sites for complex sensory integration vital for numerous cognitive processes and behaviours. The functional organisation of sensory information in the avian brainstem and cerebellum is still poorly understood. This project seeks to characterise sites in the cerebellum that receive visual and tactile signals to understand their role in translating sensory stimulation into fine motor control.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Investigating the timing and integration of sensory signals in the cerebellum will inform our understanding of neural circuits and mechanisms that control stabilisation, steering, and fine motor control. This project will also provide insight into the principles of multisensory integration that are prerequisite for biomedical applications (e.g., rehabilitation devices and prostheses) and engineered systems that must rapidly prioritise and process parallel streams of sensory information. Studying the underlying mechanisms of multimodal integration that control avian flight will have direct input for biomedical and bio-inspired technologies.

What outputs do you think you will see at the end of this project?

Outputs will include novel insights and publications related to the neuroanatomy and neurophysiology of the avian cerebellum. Characterising the neurophysiological principles underpinning avian sensorimotor control will provide insights into these conserved circuits in other species, including humans, that are involved in visuomotor behaviours.

Who or what will benefit from these outputs, and how?

In the short term, new insights into the functional circuitry of the cerebellum will guide new research aims and funding applications to support further work building on these findings.

In the long term, basic science discoveries and publications arising from this project will contribute to a broader understanding of avian flight control. These data can be used by other neuroscientists, biomedical researchers, engineers, and members of the private sector to design research questions and engineered systems related to sensorimotor control. The proposed research will inform translational research on clinical conditions, neurodegenerative diseases, and syndromes involving visuomotor dysfunction. A better understanding of the functional organisation of the cerebellum will aid researchers and clinicians in these fields.

How will you look to maximise the outputs of this work?

Outputs of this project will be maximised through internal collaborations and dissemination of new knowledge at national and international conferences. We will employ cutting edge neural recording techniques. This expertise can be applied in other animal systems, and we will form collaborations to ensure that these techniques are broadly available. National and international conferences provide important opportunities to discuss new findings and research pathways with colleagues, present data, and to establish new collaborations.

Species and numbers of animals expected to be used

- Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We have expertise identifying and recording from discrete sensory brain regions in small birds. We will use zebra finches (T. guttata), a captive species that perform rapid sensorimotor transformations. Many visual nuclei are highly conserved between avian and mammalian brains, so the work proposed here will be directly relevant to understanding critical sensorimotor pathways underlying human health and disease. Thus, birds are an effective model for understanding general principles of brain anatomy and function. Zebra finches are an appropriate model for this work because (1) they are a common laboratory model for avian neuroscience used by an active community of researchers with tools and resources for exploring neurophysiological questions; (2) my previous work has defined relevant midbrain-cerebellar visual pathways; 3) my pilot studies have identified stereotaxic coordinates for key regions and proof-of-concept for neurophysiological recordings in adult zebra finches, which reduces the chance of experimental failure.

Typically, what will be done to an animal used in your project?

Animals will undergo non-recovery surgical procedures that allow access to the brain for neural recordings. Animals will be under surgical anaesthesia for these procedures, and euthanised at the end of the relevant protocol or if signs of pain or discomfort cannot be managed with anaesthetics. Animals will be anaesthetised at the start of the procedure using an injectable anaesthetic, and depth of anaesthesia will be monitored throughout. All procedures begin by surgically accessing the brain, followed by using electrophysiological tools to identify and record from visually-responsive neurons and/or somatosensory neurons in discrete brain regions. During a subset of experiments, a pharmacological agent (e.g., tetrodotoxin and/or muscimol) will be injected into discrete sensory regions of the brainstem. Subsequently, neural recordings in the cerebellum will be performed as described above. Procedures typically last 6-12 hours but can last longer. After neural recordings are completed, the animal will be administered an anaesthetic overdose and transcardially perfused.

What are the expected impacts and/or adverse effects for the animals during your project?

Our protocols are based on well-established procedures that have undergone considerable refinement. Surgical anaesthesia will be induced at the start of the procedure. All procedures are non-recovery and anaesthesia will be monitored regularly. Animals will not suffer more than transient pain and distress and no lasting harm from handling and the initial injection, and there will be no cumulative effect from repeated injections as these are non-recovery procedures.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All procedures carried out under the proposed licence will be non-recovery procedures under general anaesthesia. Animals may be used for non-regulated procedures prior to their use on this licence.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The research addresses gaps in our understanding of how vertebrates fuse visual motion and tactile information in the cerebellum to support complex locomotion and navigation. The research requires measurement of neural activity from multisensory sites. There is no alternative to live animals for studying these sensorimotor circuits. Developing computer models to make predictions about multisensory integration requires empirically observed activity as an input. Simultaneous recordings of neural activity during sensory stimuli allows us to characterise population-level responses and inform models that will generate future avenues of research.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives that can address systems neuroscience questions about how neurons respond to sensory stimuli.

Why were they not suitable?

In all the protocols to be used, alternatives are not available that replicate the response of neurons in discrete brain regions to complex sensory stimuli or how this activity is impacted by pharmacologically blocking specific inputs.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have extensive experience performing experiments using electrophysiology in avian species, including zebra finches, hummingbirds, and pigeons. Using this experience and the large body of published literature in this field we estimated yields for (1) the number of birds in which we will find responsive cells and (2) the number of sites in each bird that will have responsive cells. Using these estimates we calculated how many birds will be required to record the appropriate number of cells.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Minimum animal numbers will be used to obtain statistically significant results, based upon power calculations, our extensive experience, and the literature. We will make every effort to improve experimental design and ensure that maximum scientific output is achieved with the smallest number of experiments consistent with scientific soundness. We perfuse all brain tissue so that immunohistochemistry studies can be performed to support other studies. Collaboration with colleagues will ensure that animal tissues are used as effectively as possible, avoiding unnecessary experimental duplication. Presentation order of visual stimuli will be randomised.

We have used the PREPARE guidelines and NC3R tools to guide our experimental design. We use power calculations and expected yield (from the literature and experience) to ensure the minimum numbers of animals are used in the programme. The following power calculation determines the minimum sample size (number of cells) required to gain power above 0.8 using the pwr package (1.3-

0) in R v4.0.3. For k = 3 groups, effect size f = 0.25, a = 0.05, power = 0.8; n = 53 cells are required for each group. Based on other neural recording studies using similar techniques in the literature, and this power calculation, we expect to need recordings from 60-100 visually responsive or tactile-sensitive neurons per region to have enough statistical power (mixed-effects regression models).

We will record multiple individual cells, across multiple recording sites, in each bird. Thus, we estimate an effective n at each level to approximate the number of animals. Based on prior experience, and a significant body of literature in the avian visual system, I anticipate requiring 20 individuals for each study to achieve this number of cells. Target size and accessibility (depth, surrounding vasculature) affects success rate for each individual (i.e., whether a target will be found). We expect to identify visually responsive or tactile-sensitive cells in 80% of birds. On average, we expect to record from 6 sites/individual (1-2 cells/site with a glass/tungsten electrode) with an expected success rate of 60% for finding visually responsive cell(s) at a given site. Thus, we expect to record 3-5 visually

responsive cells per individual. To record 80 cells, we use the following equation to calculate the number of required individuals:

(target number of cells) / (expected cell yield/individual) = number of individuals 80 cells / (0.8 * 0.6 * 9 cells) = 18 individuals

Protocol 1: We expect to need a maximum 20 birds for each study, and to perform eleven studies on this project licence (maximum 220 individuals total). Other studies in birds using similar approaches have achieved a similar yield.

We are investigating the implementation of tools that may increase the yield of each recording site -- for example, using cutting-edge neural recording technology (high-density probes; single multi-valued recording) to collect rich datasets from each individual, thus reducing the number of animals required, keeping with the 3Rs objectives. Recent studies with these probes have acquired 10-25 individual cells per site, and >200 units for multi-site network analyses. Typical recordings yield 6-10 single units per site with a 32-channel array.

Protocol 2: Up to 10 animals will be used in Protocol 2 for control tissues and setting up immunohistochemistry or other in vitro assays.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

I have expertise in performing electrophysiological recordings in zebra finches and other avian species and have mapped the locations of several visual and somatosensory brain regions. This expertise has the potential to increase the number of successful recording tracks, thus reducing animal numbers.

We are able to use relatively few animals because we rely on response properties of cells when placing our injections and determining recording sites. We will increase the yield of each recording site by using cutting-edge neural recording technology (high-density probes; single multi-valued recording) to collect rich datasets from each individual, keeping with the 3Rs objectives.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The procedures described in the proposed project are terminal and carried out under general anaesthesia, minimising pain, suffering, distress, or lasting harm to the animals.

Adult, male zebra finches will be sourced from a local commercial or other approved supplier in the UK. Zebra finches are a common avian model for neuroscience studies. The neural activity of the zebra finch song system is well-described. The project described in this licence proposal will address gaps in the literature related to the neural mechanisms involved in visual motion processing and tactile sensing. The elaborate visual system of birds and their reliance on visual inputs, rather than feedback from the ground, during flight, makes birds an ideal model for studies of visual motion processing. It is also thought that somatosensory inputs are used for flight guidance, but the mechanisms governing the processing and integration of these inputs lack significant study. These birds will also be used because of their future potential for pharmacological and behavioural studies using complex sensory stimuli as a challenge during flight. In addition, these studies will be foundational for selecting the most salient sensory cues for future studies.

Animals will be under general anaesthesia, a small craniotomy performed, and then neurons in brain regions of interest will be recorded using electrophysiological techniques while sensory stimuli are delivered (e.g., videos of moving dots or gratings, airstreams, feather deflections, etc...). Visual and somatosensory neurons respond to these stimuli in animals under general anaesthesia. Anaesthetic depth will be monitored regularly, and supplemental doses given as needed.

Pharmacological blockade via nano-scale injections of inhibitory receptor agonists or channel blockers to discrete brain regions will be used to study necessity and sufficiency of midbrain-cerebellar projections. A small injection of dye may be used to mark recording sites and then the animal will be given an overdose of anaesthesia prior to undergoing a transcardial perfusion with saline and fixative. In some cases, animals may be euthanised by a Schedule 1 method instead of undergoing transcardial perfusion.

Why can’t you use animals that are less sentient?

This is not a developmental study. Studying visual and tactile processing networks requires a fully developed neural system to understand how adult visual and tactile neural networks process information relevant for locomotion. Systems neuroscience studies, like this one, seek to experimentally open feedback loops to understand how specific stimuli affect neural activity in key regions, and how this activity impacts other nuclei within a neural pathway. The avian visual system has structures homologous to the mammalian visual system and these studies will provide insights into basic principles of midbrain-cerebellar network organisation and function that are applicable to other systems.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Local or topical anaesthetic will be used at the site of incision in addition to general anaesthesia.

For husbandry-based refinements, we will provide toys in the aviary for enrichment and cuttle bone to maintain bill health.

Handling stress will be minimal as birds typically will be handled for less than a minute prior to induction of anaesthesia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will plan, conduct, and record our experiments so that we are able to publish our results following the ARRIVE and PREPARE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will have regular contact with AWERB, the NACWO and animal technicians at the housing facility to review current approaches and whether there are any new 3Rs opportunities. AWERB and the NVS will be consulted regularly to ensure the most refined approaches are being used. LASA guidelines are being used for preparing for surgical procedures. We will use NC3R newsletters and other resources to stay abreast of advances in the 3Rs.

We have the unique advantage of wide-ranging expertise and state-of-the-art veterinary resources. We will take advantage of these resources to work with specialist anaesthesiologists to optimise protocols.

158. Intersections of Iron With Immunity, Infections, Haematopoiesis and Metabolism

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Iron, Vaccines, Immunity, Malaria, Metabolism

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to increase understanding of how iron metabolism is controlled and regulated, and of how changes in iron sensing, handling or availability affect different aspects of physiology. In particular, we aim to understand how either specific genes or altered iron states affect cellular differentiation, proliferation, metabolism and function. This will focus primarily, although not exclusively, on cells of the blood/immune system, and will investigate how iron influences the cellular output of the bone marrow, the composition of

the immune system, and immune responses (for example to vaccines, infections and tumours).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Iron is required for carrying oxygen in red blood cells, for generation of energy, and for the synthesis of DNA by enzymes, and for many other biological processes. Iron deficiency impairs these important activities, whereas too much iron is toxic. Globally, over 1 billion individuals are affected by iron deficiency, and in addition many common chronic illnesses result in iron being redistributed in the body such that it is no longer available for key processes; this can lead to deficiency in production of red blood cells for carrying oxygen (anaemia) but also likely affects many other biological systems such as the immune system - such effects are much less well understood. It is therefore important to study how genetic, nutritional and infection/inflammation-related factors influence the control and regulation of iron, and also how altered iron control or availability impacts upon the functioning of key cell types, organs and biological systems.

In particular, there is increasing evidence that a lack of iron availability for the immune system compromises its ability to mount effective and long-lasting immune responses. We want to understand how such changes in iron concentrations affect blood cells and immune responses that for example target vaccines, infections and tumours. Understanding how and why iron is important for immune cell generation, function and the downstream immune responses they participate in promises to open up approaches for improving the effectiveness of vaccines and therapies related to the immune system. This may be particularly relevant for groups and locations where iron deficiency or altered iron status is prevalent (e.g. young children and women in resource-limited settings; in the elderly; and in those with chronic conditions).

What outputs do you think you will see at the end of this project?

New information regarding the biology of iron, in particular in relation to the maintenance and functioning of the immune system.

Dissemination of new knowledge via scientific publications, national and international conference presentations, and public engagement activities.

Identification and validation of new treatment approaches may lead to patent applications.

Who or what will benefit from these outputs, and how?

Our long term intention is that the information we generate during the course of this project will lead to clinical benefit via increasing understanding of how iron interacts with the immune system and how iron status can be manipulated to either improve desired immune responses (e.g. to vaccines, or in the context of immunotherapy) or suppress unwanted immune responses (e.g. with relevance to transplant tolerance). In particular, we expect that there will be benefit for demographics frequently affected by iron deficiency (e.g. young children, especially in low-income settings) or for those receiving therapies involving the immune system. In addition, increased understanding of the interactions between iron and production of red and white blood cells will similarly benefit individuals affected by haematological disorders.

In the short term, our data will primarily be used by and of benefit to immunologists, haematologists, nutritionists (especially in the field of iron biology), other clinicians and public/global health scientists. We expect it to provide the basis and context for follow-up translational work and clinical trials in humans, with the goal of delivering on the long term aims outlined above. We also intend to present findings of the research to lay audiences via public engagement activities, increasing education and awareness of issues related to iron, vaccines and the immune response.

How will you look to maximise the outputs of this work?

The knowledge generated will be disseminated primarily via publication in peer-reviewed scientific journals; we will ensure that such publications are open access, meaning the research is available for anyone to engage with, without cost. We will also present findings at key national and international conferences (primarily meetings focussed on iron biology, immunology, haematology and nutrition).

We will also work with our communications teams, where appropriate, to disseminate broad findings more widely (e.g via press releases), and with the technology transfer team to ensure relevant findings can be patented.

Our experience is that, in addition to reinforcing existing collaborations, both publication and conference presentation frequently yield formation of new collaborations, both nationally and internationally, with groups working in complementary research areas: shared expertise and follow-up of key findings accelerates the pathway to impact of the work.

We have previously and will continue to make relevant genetically-modified murine models available to other researchers upon request, through transfer of archived frozen sperm samples.

Species and numbers of animals expected to be used

- Mice: 11000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Only mice will be used in this project. Mice are the best characterised animal species for detailed immunological and iron-related analyses, and many features of immunity, regulation of iron, and human iron-related disorders are shared or well-modelled between mice and humans. Numerous tools have been developed and are available for studying immunity in mice, meaning they can be used for detailed characterisation that is not possible in other organisms; furthermore, by using mice with defined, or inducible, gene modifications, we will also be able to study how particular genes, especially those involved in controlling iron handling, influence the immune system and metabolism.

The majority (>95%) of our experiments will use adult mice in which immune development is mature and iron status is stable. A small proportion of experiments (<5%) may involve placing juvenile mice onto altered iron diets shortly after weaning such that when they reach adulthood, experiments are not affected by the (high) iron content of standard mouse diet. A small proportion of experiments (<2%) may involve administering substances to pregnant mice and investigating effects in their embryos: this enables us to study and understand how genes related to sensing or controlling iron may influence formation of the blood system during fetal development.

Typically, what will be done to an animal used in your project?

In this project, typical experiments involve examining the immune response of mice in which iron handling or availability is altered. Altered iron handling is typically achieved by using mice carrying genetic modifications in genes linked to either blood development or iron handling, or by placing mice on a diet with low iron content, or by giving injections of substances that are known to influence how iron is handled. In many experiments, they then receive specific immunisations/vaccinations via injection. In the following days, the effect of altered iron handling or availability on the immune response may be tracked through withdrawing very small volumes of blood from the tail of the mouse and examining the effects on different white blood cells in the lab, in comparison with appropriate controls. In some experiments, the impact of iron on such immune responses may be assessed by challenging mice with appropriate infections (such as Influenza Virus, Vaccinia Virus or mouse Malaria parasites) or tumours (that are implanted by injection under the skin of the mice). Infections or tumour growth are tracked to see how well the immune response controls the infection (in mice infected with malaria, the infection is tracked by micro-sampling blood from the tail and quantifying the proportion of red blood cells harbouring parasites - this may be daily around the peak of infection); further blood samples may be taken to examine the immune response in these contexts. At the end of the experiments, mice are humanely killed, and tissues are taken for detailed immunological analysis in the lab.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect that the genetic alterations in the genetically-modified mice we use will cause more than mild effects on the mice; the majority will not be distinguishable from wild-type mice.

Our procedures for altering iron status may in some cases cause mice to become anaemic, which like in humans could be associated with fatigue and reduced movement in a small proportion of animals. In many experiments, mice are either given immunisations or substances to stimulate or alter the immune response; the effects of these are likely similar to those experienced by humans who have an acute infection, but will typically be transient (<48h): these effects may include loss of appetite (which in mice may lead to transient weight loss), reduced movement and responsiveness, and ruffling of fur. There may also be transient pain associated with receiving injections.

Mice given live infections (Influenza, Vaccinia, Malaria) are likely to develop similar effects as above as the infection develops, transiently losing weight as the infection progresses and before it is controlled by the immune response; they are also likely to show reduced responsiveness and loss of coat conditioning.

Mice with tumour cells implanted under the skin may develop palpable tumours over approximately 2 weeks, which in rare cases may impair movement.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Sub-threshold: 30%
- Mild: 45%
- Moderate: 25%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our project requires animals since the body's handling of iron and coordination of immune responses both involve multiple cell types and distinct organs/tissues. For example, a lack

of sufficient red blood cells (anaemia) is sensed by the kidney, which then sends a signal to the bone marrow to increase production of red blood cells; the bone marrow then instructs the liver to suppress the central iron hormone hepcidin, and low levels of hepcidin allow the absorption of iron from the diet and the release of iron from the spleen; the released iron increases iron availability for red blood cell production so that more red blood cells can be made, correcting the anaemia. Immune responses are similarly coordinated. Animal models, of which mouse models are the most suitable, are therefore required to dissect the mechanisms by which these processes operate and are coordinated.

Which non-animal alternatives did you consider for use in this project?

We have considered the following (which also includes indication of where we do use, or plan to use, such models)

Human observational studies: we increasingly perform either correlative, retrospective or observational studies, or sub-studies of prospective clinical trials in humans in which we can collect data on the associations of iron with immune responses and immune development.

Clinical trials in humans: we are planning to carry out clinical trials in humans, particularly those at risk of iron deficiency, to investigate whether iron supplementation enhances immune responses in humans. This clearly provides highly relevant information, but has limitations in terms of its ability to reveal biological mechanisms.

In vitro models: where possible, we look to use in vitro models, for example for basic studies of gene regulation and metabolism, or for screening compounds, thereby avoiding the need to work with animals, or reducing the numbers of animals that would otherwise have been required. For example:

1. Human white blood cells, isolated from human blood: we are pursuing this as a model for investigating how low iron concentrations affect mechanisms of energy usage by white blood cells.

2. Human erythroid differentiation cultures: we have been developing and optimising a knockout model in human primary cells to aid investigation of a gene closely linked with red blood cell development, and its relation to iron and nutrient sensing; this model means a significant proportion of experiments can be carried out in a human in vitro model, with an accordingly smaller proportion of work being carried out using a corresponding mouse knockout model.

3. Immortalised (cancer) cell lines: these are straightforward to work with and can be useful for basic investigations of iron regulation:

example 1: studies of how the iron hormone is regulated in liver-derived cells, and screening of potential therapeutics

example 2: use of immortalised immune cell lines

In silico analysis of pre-existing datasets: Where possible/available, we screen literature and datasets for relevant pre-existing large biological datasets from which we may extract and infer information regarding how iron-dependent processes may interact with, for example, the function of immune cells.

Why were they not suitable?

Human observational studies and clinical trials: while such studies can and do provide key information describing relationships between iron and the immune system in humans, in our context they are typically based on analysis of blood (due to its ease of accessibility). However, key aspects of the immune response take place in specialised immune tissues that are not easily accessible in humans, but which are amenable to investigation through mouse models. Importantly, mouse models additionally facilitate better controlled investigations of immune mechanisms due to the availability of inbred strains, as opposed to genetic heterogeneity in human studies.

In vitro models: although we can and do gain important insights from our in vitro experiments, since the aspects of physiology we are investigating are multi-organ systems or dependent on specific tissue organisation in vivo, it is necessary to validate the relevance of such insights in a whole animal (mouse) model. For example, even though we may identify key biological pathways influenced by iron in T cells, the cells in vitro grow in a different medium compared to cells in vivo. Furthermore, when considering immortalised cells, it is frequently the case that the process of immortalisation interferes with the cellular processes that we wish to understand, limiting their relevance.

In silico models: findings from analysis of large pre-existing datasets require validation, which may be done initially in vitro, but ultimately in vivo, so their stand-alone capability remains limited - especially their ability to reveal biological mechanisms.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers of mice estimated here reflect our projected course of work for the next 5 years and the experiments likely required to achieve the stated objectives. This estimate takes into account experience from our previous licence which has covered the last 5 years as a guide, and therefore encompasses typical experiment size, typical duration of experiment,

and the likely number of individuals working in the lab who may simultaneously be working under this licence over the coming 5 year project licence period.

Experiment groups are designed to be of sizes such that evidence for biologically meaningful effects can be detected and reported with confidence; this frequently involves performing replicates of experiments to ensure robustness of data. Experimental mice will either be bred under this licence (genetically-modified strains) or purchased from commercial vendors or core facilities (typically for wild- type strains) with authority to supply mice: the estimate for numbers of mice on the breeding protocol is based on our previous experience and reflects the number of genetically modified strains likely to be required, mice required for colony maintenance, mice of non-desired genotypes, and breeding required for supply to other researchers for example.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

1. Good experimental design (based on prior data on typical experimental variability) allows selection of group sizes appropriate for testing experimental hypotheses (thereby avoiding the risk of needing to repeat experiments due to insufficient mouse numbers in relation to the degree of variation in data (underpowering), or use of unnecessarily high large sizes).

2. Where possible, we collect secondary outcome data from animal experiments to avoid the need for separate experiments using additional mice. To maximise the range of data that can be obtained from single experiments, we routinely use a team of 2-3 lab members to process tissues / prepare samples

– this allows for collection of a broader array of tissues and increases the efficiency and quality of data collection, thereby reducing the number of experiments that might otherwise be required for each objective. Thus, even if experiments have a key primary outcome, it is usual that multiple secondary outcome tissues/data are collected from each experiment.

3. Past experience enables selection of experimental time-points that maximise the amount of data while using the minimum number of interventions; where possible/appropriate, longitudinal sampling from the same animals across a time course, e.g. blood sampling during an immunisation timecourse can also lead to reductions in overall numbers of mice required, and enhance data quality.

4. Tissues are frequently archived, such that in principle, alternative/related outcomes may also be investigated at a latter date without the need for additional experiments on new mice.

5. Where possible, experiments are performed using both male and female mice from breeding protocols, with appropriate statistical approaches to account for sex-specific

differences employed in the analysis. This has the effect of reducing the numbers of breeding pairs/trios required to reach required group sizes.

6. Where possible, we will look to employ experimental strategies that can reduce the overall numbers of mice required (even if not formally a 'replacement'); for example through efficiently employing ex vivo approaches:

we have been optimising in vitro culture models for murine malaria parasites, which require mice as a source of red cells to be infected by the mouse parasites and to produce stocks of parasites. Rather than setting up mice separately to generate these, where possible, we aim to schedule in vivo and in vitro experiments simultaneously to allow us use donor mice that have been prepared for in vivo malaria infections to also provide red cells and parasites for in vitro experiments – this allows an overall reduction in numbers of mice required.

we will look to use 'ex vivo' culture models where appropriate for studying iron-immune cell interactions: spleens or lymph nodes are taken from wild-type or genetically-modified mice that have not undergone procedures and are used as source of desired immune cell types for isolation and culture under altered iron conditions ex vivo. Such experiments can complement the in vivo experiments, and overall allow a reduction of about 80% in mice required compared to equivalent experiments in vivo.

7. We will archive genetically-modified lines (for example by sperm freezing) in a timely manner such that breeding can be stopped when sufficient data has been obtained, while retaining the potential to re-establish them if required: this will avoid unnecessary tick-over breeding of mice that are not required for experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

1. Members of our group have received specific training from colleagues with years of experience of colony management in how to efficiently run and manage breeding colonies; we will encourage repeat of this training for new lab members. Employing these recommended approaches - including archiving of mouse lines, backcrossing, estimation of appropriate numbers of breeding cages to generate required numbers of mice in a timely manner - reduces wastage of animals and mitigates against the risk of genetic drift which may confound experiments necessitating repeats.

2. For experimental conditions where we have no prior experience or for which there is no precedent from colleagues, collaborators or published literature, we will carry out pilot studies - from these we can examine the expected variability in desired outcome measures, such that appropriate group sizes for main experiments can be estimated.

3. Where appropriate, fresh or frozen tissue from relevant colonies/experiments will be shared with colleagues and collaborators (including both donations and receipts of material).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have chosen mice for this project as the best characterised and least sentient relevant species for detailed analysis of iron biology and immune function. In our experiments, as soon as the scientific objective(s) of the procedure(s) has been achieved, or a humane endpoint has been reached in line with specific and well-defined monitoring guidance, mice will be humanely killed, such that lasting or unnecessary harm is not caused. In order to evaluate the effects of altered iron availability on immunity and infection, we need to robustly assess impact against infection and therefore mice may experience illness in some experiments; however, mice will be monitored daily and as infections progress, the frequency of monitoring will increase to ensure undue suffering is not occurring. In most models we use, such suffering - if apparent - is expected to be transient, as inflammation following stimulation with inflammatory agents typically resolves quickly, or infections are cleared rapidly by the immune system.

Humane endpoints will be constantly re-assessed and refinements implemented to minimise systemic illness while maintaining the scientific quality of the study.

The following specific measures will be taken to cause the least pain, suffering or distress:

1. Genetically-modified mice: we expect the genetic modifications in the models we use to yield no more than mild, and in the majority of cases, no apparent clinical effects on mice.

2. Substances: The substances we use are typically well documented and we have extensive experience using them. We give the smallest dose possible to create the effect needed thus reducing the harm to the animal. Pilot experiments will be carried out to assess the effects of substances for which we or others do not have prior experience.

3. Optimal analysis of immune responses: We will use our previous experience, and evidence from the literature, relating to typical kinetics of immune responses, to ensure that blood samples for analysis are only taken at the most informative time points.

4. Adjuvants: For experiments that involve inducing immune responses or vaccines, we will use non-ulcerative adjuvants (or bioequivalent); Freund's adjuvant will not be used in our studies.

5. Irradiation: Use of irradiation to deplete the immune system is favoured over the use of chemical agents as the cumulative effects are less severe. When irradiating mice to ablate bone marrow cells for the production of bone marrow chimaeras, split dose irradiation is used to focus DNA damage on the dividing cells in the bone marrow and reduce the need for higher doses of irradiation that would cause damage to other tissues.

6. Infection models: the models we used are well characterised and we have previous experience of working with them; they are expected to cause transient clinical effects from which the mice are expected to recover. The malaria parasite species / mouse strain combinations we will use are self-limiting non-lethal infections in wild-type mice; we will avoid parasite species associated with a higher risk of lethality. The doses of influenza viruses are calculated to achieve complete infection of all control animals, while minimising lethality. Well-defined monitoring requirements and humane endpoints are applied to minimise the risk of harms from our infection models.

7. Tumour models: the tumour models we will use, in which tumours are implanted under the skin, are straightforward for non-invasive monitoring, and risk of secondary tumours (metastasis) is very low.

8. Anaesthesia: where possible, steps requiring anaesthesia will be combined.

Why can't you use animals that are less sentient?

Mice are the lowest appropriate species for studies of mammalian iron biology, immunity and infection pathogenesis. It is important that our models recapitulate the major features of the human immune system and mechanisms of regulating iron: unfortunately key aspects of both iron regulation and the immune system are not shared in protected non-mammalian species such as zebrafish or non- protected species such as fruit flies. In addition, mice are the best characterised species for detailed immunological/infection and iron-related analyses: because of the numerous different immunological tools available, mice can be used for detailed characterisation which is not possible in other organisms (either more or less sentient).

Our experiments involve adult mice, in which time has been given for the immune system and iron status to develop, mature and stabilise, which is important for interpretation of experimental data. Almost all experiments use young adult mice however (i.e. starting with 6-8 week old mice) and the majority of protocols are concluded within 4 weeks of initiation;

however, these protocols will last longer than would be feasible under terminal anaesthesia, meaning that is typically not an option in our experiments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will frequently review and refine the doses, schedules and frequencies of substances administered to ensure minimum welfare impact on the animals, whilst still facilitating collection of meaningful biological data.

Where appropriate scientifically, we will use routes of substance administration associated with least pain/distress (e.g. via drinking water rather than repeated gavage).

In cases where weight loss is expected to be associated with a procedure, and where it will not have a significant scientific impact on the experiment or data collected, moistened palatable food will be offered when mice that have lost over a pre-defined proportion of their starting weight.

We will use the most refined methods for blood sampling, which is particularly relevant for our malaria experiments in which small volume sampling may be required daily: for this, needle puncture of the tail vein will be used to obtain the <5µl sample volume required.

If genetic modifications, altered diets, or treatments are found to be associated with increased urination, bedding will be altered to increase absorptive capacity.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure experiments are conducted in the most refined way, we will use guidance from bodies including:

NC3Rs: https://www.nc3rs.org.uk/3rs-resources

https://norecopa.no - including the PREPARE guidelines for planning animal experiments
https://www.lasa.co.uk

We will also consider additional guidance and direction provided by our Named Information Officer / 3Rs Information Officer, working through relevant implications and implementation of such guidance with Vets and animal welfare officers where necessary.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Individuals working under this licence are required to attend the regular animal welfare meetings organised by the department, at which vets and animal welfare officers regularly speak, providing updates on advances in 3Rs; recommended changes and advances are then discussed within our group, and if necessary through further discussion with vets and

welfare officers to facilitate application. Updates from NC3Rs are also regularly disseminated by the designated 3Rs information officer. Information is also provided by the NC3Rs regional manager, and we will check for relevant updates on advances from the NC3Rs webpage (https://www.nc3rs.org.uk/) and other related webpages/resources.

159. Electrical Impedance Tomography of Nervous System Function

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Electrical impedance tomography, Epilepsy, Cognitive neuroscience, Medical imaging, Biomedical engineering

Animal types	Life stages
Mice	adult, aged
Rats	adult, aged
Rabbits	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To continue development of a new medical imaging method that, for the first time, enables the imaging of nervous activity in the brain or nerves in 3D. The technique is Electrical Impedance Tomography.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Over the past two decades, there have been great advances in medical imaging, with the development of techniques such as a functional MRI or positron emission tomography which enable imaging of activity in the brain. However, this is only of slow changes over seconds related to increased metabolism or bloodflow. There is currently no method which enables imaging in 3-D of fast neural activity over milliseconds in circuits in the brain or nerves. Electrical impedance tomography (EIT) is a new medical imaging method that, uniquely, offers this possibility. Imaging of fast or related slow nervous activity is possible with electrodes arranged around the edge of the organ of interest. Our group has pioneered this method and demonstrated proof of principle for imaging activity both in the brain and also in peripheral nerve such as the cervical vagus nerve in the neck. This has huge potential for better understanding of the physiology of breakdown nerve circuits and improvement of the management of diseases such as epilepsy in the brain or disease treatment by electrical stimulation of nerves.

The original vision was that this could be accomplished noninvasively with electrodes placed on the scalp to image brain activity. However, for technical reasons, the application in the brain has been only possible with intracranial electrodes placed inside the skull either on the brain or with penetrating electrodes descending into the brain matter itself. In addition, we have so far only been able with this approach to image changes in the outer third of the brain. We are actively working on extending the technology so that we would be able to image circuit activity in the brain noninvasively with scalp electrodes and the new technique of Atomic magnetometers. We are also working on being able to image activity anywhere in the brain or in nerves using ultra-thin flexible electrodes implanted into the brain or peripheral nerve. If successful, this will provide a groundbreaking new technology suitable for use in humans for better imaging and treatment of cerebral disease conditions. It is possible to undertake some of research using computer modelling and studies in liquid filled tanks. However, to develop the technology to the state where it could be used in cognitive neuroscience, autonomic nervous system research, human medical subjects, it is essential to undertake development work in experimental animals in vivo.

What outputs do you think you will see at the end of this project?

The principal benefits will be the development of methods to a point where they can be used in human clinical trials. Specifically, this will be development of technology with intracranial electrodes for use in human subjects undergoing presurgical evaluation for epilepsy, a non-invasive electrode and atomic magnetometer helmet in human subjects for studying cognitive neuroscience and improving epilepsy management, and an EIT nerve cuff suitable for implantation in the cervical vagus nerve of human subjects for treatment of conditions such as epilepsy, diabetes mellitus, or rheumatoid arthritis. The work will be

published in scientific journals and will also form the basis for technology to be made available to neuroscientists through a spin out company based in the laboratory.

Who or what will benefit from these outputs, and how?

Human subjects with conditions such as epilepsy, schizophrenia, depression, stroke, diabetes mellitus, or rheumatoid arthritis will benefit in clinical trials using the EIT technology if it can be shown to be successful. Neuroscientists who are interested in advancing understanding of circuit activity in the brain or in peripheral nerves will benefit by having a new uniquely advantageous method for localising fast neural activity or other pathological conditions in experimental animals or human subjects. These benefits will be available in the short term after about 2 to 3 years for neuroscience in experimental animals and in the medium term of 5 to 10 years for human disease conditions which require clinical trials.

How will you look to maximise the outputs of this work?

1. Publication in peer-reviewed scientific journals.

2. Collaboration with internationally leading groups in the development of the technology and in use of the developed technology.

3. Successful grant applications.

4. Pre-registered reports.

Species and numbers of animals expected to be used

- Mice: 100
- Rats: 300
- Rabbits: 50

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult rodents and rabbits will be used because they have been the species of choice in previous studies both in the literature and in our own work for the targeted brain and nerve activity such as normal function in the cerebral cortex, peripheral nerves, or models of epilepsy or stroke.

Typically, what will be done to an animal used in your project?

All studies will be terminal in which the animal is anaesthetised and does not recover. EIT imaging will be undertaken using electrodes placed on the scalp, on or in the brain, or around peripheral nerves while activity is produced by physiological stimulation for production of simulated disease conditions such as epilepsy or stroke. Activity of the brain or nerves may be modulated by electrical or pharmacological modification.

What are the expected impacts and/or adverse effects for the animals during your project?

Anaesthesia will be induced by painless standard methods, usually just by painless inhalation of anaesthetic vapour and thereafter animals do not experience any pain as they do not recover.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The studies will be undertaken in terminal experiments in rats, mice, or rabbits which severity is non- recovery as the animal does not recover after induction of anaesthesia.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The method has small signals which are at the limit of detectability. Previous experience has shown that optimisation of technical parameters is only possible under laboratory conditions in experimental animals where prolonged recording and adjustment of variables is possible. As soon as this can be accomplished, we plan to move to human studies.

Which non-animal alternatives did you consider for use in this project?

1. It is possible to undertake preliminary studies with computer modelling or fluid filled tanks.

2. Human studies.

Why were they not suitable?

1. Computer modelling fluid filled tanks are useful in developing the methodology but cannot produce the underlying physiological or pathological conditions encountered in mammalian tissue and also the confounding variables which occur when measurements are made in vivo.

2. Human studies are restricted in the case of intracranial electrodes to very limited specialised cases where these have been inserted for other purposes such as presurgical evaluation for epilepsy, or insertion of stimulating nodes cuffs around the vagus nerve in the neck for treatment of epilepsy. These do not enable modification and refinement of technological variables such as electrode design or improvement of hardware or software; these are only possible in repeated animal studies with interactive refinement. It is also essential to have preliminary data in animal studies in order to obtain ethical approval for human studies

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

There are several different technological approaches which are listed in the plan of work. From experience of working in this field over many years, I have estimated the number of animals needed for the stages of iterative refinement and a collection of data in approximately 5 to 10 animals needed for statistical justification of the finished technology for different iterations of the technology.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In all experiments, we undertake constant review to ensure that only the animals needed to achieve technical goals of satisfactory signal and image quality are used. All projects are discussed with senior statistical advisers to decide on the minimum number needed to provide statistical significance of the accuracy of the images. The data produced comprise EIT imaging data sets which are compared to independent gold standards such as MRI or CT for the brain or measurement of nerve or brain activity with electrodes. The data are usually analysed objectively using a standard statistical method for medical images, Statistical Parametric Mapping.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As above.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The studies are terminal so there is no suffering.

Why can’t you use animals that are less sentient?

Rats, mice and rabbits have been selected because they have been extensively investigated for such changes in the literature. Their brains are of a reasonable size for surgery but they are still small laboratory animals and so relatively easy to manage. Adults have been chosen because there are technical challenges with the use of smaller electrodes in immature animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Not applicable for terminal studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In all cases, our experimental preparations follow published methods in the physiological literature. We will follow the latest and most refined methods in scientific publications in refereed journals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Members of the research group will actively engage with biological services at our institution and keep abreast of the literature with respect to these advances. They will be actively discussed at regular meetings to discuss progress on the project. I will liaise with our local regional NC3Rs manger and plan to attend additional training e.g. Bioscience Research Consultancy | Responsible Research in Practice

160. A Mouse Model of Multiple Myeloma

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, myeloma, NSD2, mouse, blood

Animal types	Life stages
Mice	juvenile, adult, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To develop and test a novel genetically engineered mouse model for the cancer multiple myeloma.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Around 1 in 100 people will develop multiple myeloma (MM) in their life time, equating to 6000 diagnoses in the UK every year. Despite advances in patient treatment over the last

twenty years the disease remains essentially incurable and only 33% of patients are alive 10 years after their diagnosis. Understanding the genetic basis of how myeloma develops and evolves is crucial for rational drug development. Currently there are no mouse models of myeloma which adequately mimic the underlying disease biology. We have therefore developed a novel genetically engineered mouse of model which mimics MM biology. It is important to develop this model as it will afford opportunities to better understand of how the disease develops and test novel therapeutics.

What outputs do you think you will see at the end of this project?

The initial benefit of this project will be the creation of a genetically modified mouse model of multiple myeloma which faithfully recapitulates human disease biology. This system can then be used to both understand how the disease develops and test of novel therapeutic agents to improve patient treatment. We will also make our model available to the wider scientific community for others to exploit.

We will analyse the genetic profiles of tumours potentially yielding insights into disease biology, these findings will be communicated in scientific publications.

Who or what will benefit from these outputs, and how?

Improving our understanding of myeloma biology and the testing novel drugs has been restricted by absence of appropriate animal models. In the short term this genetic model system aims to provide insights into disease biology benefiting scientists. In the longer term we aim to provide benefit for myeloma patients by accelerating drug development.

How will you look to maximise the outputs of this work?

Our findings will be communicated via publication in scientific journals.

We will make our genetic model available to the scientific community in conjunction with appropriate 3rd parties.

Species and numbers of animals expected to be used

- Mice: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The development of myeloma cannot be modelled in cell lines. It is necessary to use animals because they more closely mirror the normal biological environment in which cancer develops, for example including different cell types. Myeloma cells from patients

cannot generally be grown or cultured long- term. While cell lines of myeloma do exist they are not considered to faithfully reflect the disease. As myeloma is a disease of plasma cells (a type of B-cell) we cannot use other commonly use animal models (e.g. zebrafish or flies). These animals don't possess the same antibodies that plasma cells produce and therefore are not analogous to mammalian species. Our proposed programme of work will utilise other pre-existing genetically engineered murine models. Consequently, there is no viable alternatives to mice.

Procedures will be performed on adult mice as the disease is not expected to manifest in early life.

Typically, what will be done to an animal used in your project?

Our novel genetically modified mice will be bred with other pre-existing genetic models producing different genetic backgrounds, each addressing an important question relating to myeloma development.

Animals with desired genetic backgrounds will be monitored for signs of disease for up to 18 months. During this period every three months we will perform two procedures. Firstly, animals will have a blood and urine sample taken. Secondly, animals will be anaesthetised and imaged to visualise tumour development.

What are the expected impacts and/or adverse effects for the animals during your project?

We anticipate that some animals will develop myeloma, the adverse effects of which are likely to include weight loss and pain. These harmful phenotypes will only be experienced in a subset of the animals in the project. The genetic modifications being used are in an inactive state until they are found in combination with an activating genetic change. We will restrict this from occurring until we are ready to study the disease. This will prevent animals in breeding colonies from experiencing harmfully effects due to genetic changes. The severity of harmful phenotypes will be limited by using defined humane endpoints, which, when reached will trigger euthanasia.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We anticipate animals developing cancer will experience a moderate level of severity.

We expect such animals will show signs including, reduced activity, reduced feeding and increased thirst.

We expect 100% of animals in the breeding protocol to experience a maximum of mild severity.

We expect 50% of animals in the monitoring and imaging protocol to experience a moderate severity. The reminder we expect will experience a mild severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Multiple myeloma cannot be modelled in cell lines. It is necessary to use animals to provide the multi- cellular environment required to model these processes. Furthermore, development of novel therapeutics requires animal models that faithfully recapitulate the biological process promoting human disease. A main project aim is to create and validate such a model system, consequently we must utilise animals.

Which non-animal alternatives did you consider for use in this project?

Human multiple myeloma cell lines.

Why were they not suitable?

The development myeloma cannot be modelled in cell lines.

Drug testing can be performed in cells lines, however promising candidates must be validated in animals model systems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Statistical robustness is linked to disease frequency in test animals, the greater this frequency the lower the number of animals required to demonstrate an effect. We have calculated the number of animals required by estimating the number of animals we expect

to develop disease in each arm of the experiment. Based on these estimates we have chosen the smallest number of individuals required to be sure there is difference in disease incidence in different experimental arms.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our experiment was modelled using the NC3Rs experimental design assistant. We have also had the design assessed by in house statisticians and animals welfare personnel within the Institute.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have designed a breeding strategy which utilises 100% of the animals once breeding animals have been generated, helping to reduce the numbers euthanised due to genotype.

As we cannot predict the disease incidence in experimental test arms, rendering estimation of optimal study size challenging. We have therefore included an additional experimental arm which we predict will increase disease incidence increasing our ability to detect an effect and therefore requiring less animals.

We are collecting and archiving tissue from all test animals, while this increasing labour in this study, the measure will prevent the need to conduct follow up experiments for this purpose.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use 4 different strains of genetically modified mice, all strains are bred on the C57BL/6 inbred genetic background. We have used this model as it is necessary to preform our experiments in species producing the IgG subtype of antibody.

We have developed a novel genetically modified mouse stain for the experiment. This strain will produce an excess of a particular gene product, associated with the development of myeloma. Excess production from this gene and one other gene, produced in another genetically modified strain we are using, could have undesirable

consequences, for example the development of cancer in a tissue other than the blood. We have therefore designed our experiment such that genetic alterations will only be experienced in plasma cells. This approach has two key benefits, firstly animals should only acquire myeloma and not another cancer and secondly when breeding can we prevent animals from unnecessarily developing disease. The combined effects of this refinement reduce the number of animals required and reduce the suffering of those remaining.

We don’t anticipate that any of the planned procedures will cause anything other mild and transient pain, suffering or distress to the animals. However to reduce pain and distress to animals we will perform, where possible, our 3 monthly imaging and blood collection in one session under general anaesthetic.

We do anticipate that some of the animals will develop myeloma, consequently we will monitor various criteria to detect pain and distress. When these criteria are met animals will be euthanised preventing further suffering.

Why can’t you use animals that are less sentient?

Faithful modelling of myeloma requires a species produce IgG antibodies. Other commonly use animal models (e.g. Zebrafish or flies) do not have a complex immune system and do not produce plasma cells analogous to mammalian species. Consequently, there is are viable alternatives.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will impliment new animal welfare best practice when made aware from sources such as the NC3Rs, Arrive guidelines, LASA etc.

The main welfare implication of our study is the development of cancer in some animals. We will examine whether, animals developing cancer, could be euthanised earlier prior to human endpoints criteria being met. It may be possible to identify early indications disease sufficient to satisfy study objectives before the onset of discernible signs

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs, Arrive guidelines, LASA.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

With reference to the N3Rs website, with whom I have registered and receive regular updates.

Suggested best practice changes, impacting our study, will be discussed with our NACWO and where relevant NVS to determine whether revisions to our protocols are necessary. In this event we will, submit an amendment to our licence protocol to reflect their adoption.

161. Control of Neural Stem Cell Proliferation and Differentiation

Project duration

2 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Stem cells, Neurobiology, Neurological disease, Cell biology, Metabolism

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

During development a founding population of precursor cells, called neural stem cells, generate all the neurons (cells that relay information) and the supporting glial cells (which provide nutrients to the neurons and insulate them, much like the wrapping of electrical cables) of the central nervous system in a precisely controlled manner. Central to this process is the transition of the neural stem cells from their precursor, functionally unspecialised state to a differentiated (functionally specialised) state.
Cellular metabolism together with protein synthesis are crucial components of such decisions. The aim of this project is to elucidate the metabolic mechanisms responsible for neural stem cell differentiation in order to understand how these control normal neural stem cell physiology and how their de- regulation contributes to neurological disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Metabolism in neural stem cells is controlled by the "powerhouses" of the cells, called mitochondria. Both mitochondrial dysfunction and aberrations in protein synthesis are hallmarks of all neurological diseases. There is a tremendous need to understand these basic mechanisms in healthy neurons in order to uncover what goes wrong in pathological conditions and be able to target these mechanisms therapeutically. At present, there is very little that can be done medically to treat neurological diseases, yet the suffering and societal burden of these is immense. Therefore, any new knowledge we gain about both normal and pathological function of mitochondria and protein synthesis will put us in a stronger position to target these therapeutically in the longer term.

What outputs do you think you will see at the end of this project?

At the end of this project, we expect that have several scientific publications given the novelty and importance of the topics. Moreover, I have set up new collaborations with clinicians working on ALS/FTD (amyotrophic lateral sclerosis/fronto-temporal dementia) and will investigate the relevance of the biological processes we are studying in these diseases. It is anticipated that there will be major contributions to our understanding of these diseases with the topics we are addressing as they are highly de-regulated in ALS/FTD.

I am in receipt of recent funding obtained from the Gerald Kerkut Trust to carry on with the project (£20k). There is also a collaborative grant application to the NIH with very high scores, so this is likely to be funded in the next 6-8 months and at that stage I will share results with the scientific community. This is why I have initially requested that the duration of the PPL be for two years with the possibility of applying for a further 3 years duration once additional funding has been obtained.

Who or what will benefit from these outputs, and how?

In the short term (within the next year), the scientific community will be the primary beneficiary of the findings through upcoming submission of a manuscript with the latest research discoveries. However, in the longer term we anticipate that there will be substantial contributions to our understanding of clinical relevance of the newly gained mechanistic insights in Amyotrophic lateral sclerosis (ALS)/Frontotemporal dementia (FTD) disease at least, given our collaboration with the clinical researchers in the field and access to patient material for analysis. Gaining the understanding of clinically relevant mechanisms will put us in a strong position to begin designing targeted therapies to correct the aberrations observed in order to alleviate some of the immense suffering ALS patients endure. It is unlikely that any such designs could be brought about even by the end of the project period, but it is likely that it will become clear which components of the de-regulated system are best placed to be manipulated.

How will you look to maximise the outputs of this work?

I will look to maximise outputs by publishing my work in peer reviewed journals and presenting it at conferences. However, should we discover that the mechanisms are relevant to ALS/FTD, we will liaise with the relevant charities to inform them of our findings and will set up information presentations for patients and their families.

Species and numbers of animals expected to be used

- Mice: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The large number of different types of neurons and glial cells that make up the central nervous system (CNS) all develop from "neural stem cells" (NSCs) in the embryonic neural tube (forerunner of the CNS). NSCs in different parts of the neural tube come under the influence of different signalling molecules from local "signalling centres", which direct the NSCs along different developmental pathways. Most of the molecular signals have not been identified yet so it is not feasible to attempt to study CNS development solely in tissue culture. It must be studied at least partly in intact animals, where the signalling centres are not disrupted. Mice are the animal of choice since they are the only mammalian species that can be easily manipulated genetically, and for which brain anatomy, physiology and function has been well documented. Many of our experiments on e.g.,, regulation of NSC differentiation during development will be carried out using cultured cells - either established cell lines that have properties of neural stem cells (e.g., P19 mouse embryonal cells) or primary neural stem cells derived from embryonic or adult mice after humane killing.

Typically, what will be done to an animal used in your project?

Mainly, the animals will be used for breeding and maintaining both genetically altered and control strains over the period of requested project licence. Resulting pregnancies will be either terminated by humane killing of the mothers at any stage up to term if embryos are required for analysis or allowed to run the natural course to term. Following identification of genetic status, animals may be maintained for conventional breeding or used for the studies proposed. Tissue biopsy will be obtained by a small ear punch to determine genetic status of the animals. We will maintain some animals for up to 15 months to investigate any potential abnormalities in the brains of older animals as a result of mutation introduced.

In order to assess how the neural stem cells from our transgenic mice are proliferating or what the rate of protein synthesis is in the genetically modified mice, we shall administer substances that allow us to measure these processes in vivo. This will be done using pregnant female mice (if assessing embryonic NSC proliferation or protein synthesis rates) or adult mice, by injecting substances using the least invasive recommended route (e,g. subcutaneous route) for a defined period of time. For studying protein synthesis, a single injection is enough to detect the signal and provide the information necessary to quantify protein synthesis rates. For measuring proliferation of neural stem cells, the length of administration will depend on the specific question being addressed, but is unlikely to exceed three injections in total.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not anticipate a harmful phenotype to genetically altered animals. From our experience and published research, mice are not expected to show any suffering or

distress, do not develop any abnormal cell growth like tumours or show any abnormal behaviour and animals remain fertile. We noticed that genetically altered males may gain weight compared to control litter mates, suggesting a mild metabolic dysfunction. However, they are reproductively active and do not exhibit any behaviours indicative of suffering. We will maintain some animals used in our experiments for 15 months to investigate any potential abnormalities in the latter as they get older.

Ear notching for biopsy collection and genotyping of animals should involve only mild and transient pain, with no healing problems.

There may be mild discomfort when administering substances to mice (both pregnant and not pregnant ones). We endeavour to minimise discomfort as such injections are administered by experienced and fully competent personnel. Substances used in outlined experiments are safe and commonly used to study proliferation rates of dividing cells and protein synthesis rates. Neither of the two substances used has been shown to cause adverse reaction at proposed concentration range and duration of administration outlined in the experiments.

Should we observe anything unusual in the behaviour of our animals that suggests suffering or discomfort that may progress beyond transient and moderate, animals will be humanely killed following well-established humane experimental points.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most of the procedure listed on the licence are of mild severity (70%); some animals may receive repetitive dosing of substances and/or temporary loss or gain of body weight which may cause a temporary mild and /or intermittent moderate distress for mice.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

All neurons and their support cells, called glial cells, that make up the central nervous system (CNS) develop from "neural stem cells" in the embryonic neural tube - the structure which eventually develops into the brain and spinal cord. Neural stem cells receive local instructions from different parts of the neural tube, which direct them along different developmental pathways. The molecular nature of these signals has not been identified in detail making it practically impossible to study CNS development in tissue culture only. This necessitates the use of intact animals for such studies to be truly informative.

Which non-animal alternatives did you consider for use in this project?

We use cell lines with properties closely resembling those of developing neural stem cells when possible. In the longer term, I plan to set up organoid cultures (often called mini-brain in a dish) from human cells to study metabolic pathways affected in neural stem cells and other neural populations where the gene whose function we are studying is deleted.

Why were they not suitable?

As mentioned above, at the moment, even organoid systems, that are probably the closest experimental model that allows to follow developmental trajectories of various organs and avoids the use of animals, are lacking in the full complexity of a whole organism with various tissues influencing each other's development. While much progress has been made and we are following these developments of refined organoids closely and have already discussed possibilities of collaborating with the world leading experts in the field, it is still not a complete replica of the real embryonic development that an animal, such as mouse, offers. Moreover, it is impossible to perform behavioural experiments to assess, e.g., learning and memory, using such organoid systems at present, thus necessitating the use of whole animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimate is based on our previous usage and the projected usage during the tenure of the proposed studies for this PPL which may include behavioural testing of genetically altered mice. Behavioural testing will require use of higher numbers of animals to achieve statistical significance.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Many of our mechanistic studies will be performed using established immortalised cell lines and only verified in primary neural stem cells or animals, thus minimising the numbers of animals used.

To quantify cell numbers, we use multiple brain sections from around 5 animals per experiment. Using litter mates and performing independent experiments in duplicate or triplicate minimizes and controls for inter-animal variation, in the process helping to reduce the number of litters used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have already generated a transgenic mouse strain which carries the deletion of the gene of interest, from the neural stem cells and produces a Green Fluorescent Protein which can then be used to study affected cells. This fluorescent tool is safe and extensively used and will allow us to reduce the number of breeding crosses necessary for our proposed experiments and minimise the total number of animals used overall.

We have established much of the methodology we use in the proposed experiments during the period covered by the original PPL and are therefore in a position to proceed with experiments efficiently since we know the parameters necessary to obtain relevant information (for example the timing of administered substances required to obtain desired and interpretable results). This will minimise the number of animals we will use to answer the questions we are addressing.

We will share tissue with any other research groups interested in using it to minimise the number of animals used in experiments. We have already provided embryonic brain tissue to colleagues investigating various mechanisms of brain development and advised them on the most efficient use of tissue to obtain neural stem cells.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Throughout this project, we will be using genetically altered mice. These will be used for breeding to maintain our ongoing colony of mice. Moreover, since we are studying how the brain develops during development, we will use these mice to generate embryos for investigation of brain development at specific times during gestation. Therefore, mice will be time mated (a pairing set up overnight and a male removed from the female cage in the morning) and females will be humanely killed at specific time points to obtain embryos. Humane killing will be carried out using the recommended Schedule 1 procedure to minimise distress. We normally isolate tissues from the sacrificed females to use in biochemical experiments thus ensuring maximal use of the animals in the study.

We also administer substances to our genetically altered mice to assess the rates of neural stem cell proliferation and protein synthesis rates in the developing embryonic tissue. For proliferation measurements we use substances that are used routinely for such studies for a considerable time without any indication of adverse reactions in mice (including in pregnant animals) when applied over the length of time proposed here (maximally over a period of three days). For most of our studies, animals will be humanely killed 1-2 hours post-period of injections to isolate embryos and process tissue for analysis.

Similarly, to measure protein synthesis in vivo, commonly used substances will be used to visualise and quantify protein synthesis rates, including in pregnant females. We have

established that a very short pulse time (<30min) following dosing to provide a robust signal with clear results that can be quantified in tissue. At the end of the procedure, animals will be humanely killed by Schedule 1 method.

While some distress is inevitable when administering substances to animals, this will be kept to a minimum by performing these procedures quickly by trained personnel. The outlined procedures will not cause lasting harm to the animals used because they are humanely killed at the end of such experiments.

Why can't you use animals that are less sentient?

Most of our research centres on the embryonic development using mice as the model animal. However, in order to obtain embryos, we must use pregnant females. Throughout the project, we keep the number of pregnant females that are sacrificed to an absolute minimum to ensure statistical significance of our results. Each female of the mouse strain we work with has a litter size of 6-8 embryos on average, thus providing us with sufficient number of embryos for reproducibility within each litter. Moreover, out breeding strategy allows us to obtain litters that have only mutant and control embryos thus minimising inter-litter variability and the number of breedings we need to set up. In this way we make most use of our breeding strategy to ensure the use of the lowest number of animals necessary for our study and the least distress to adult mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

At the moment, mice are the animal of choice since they are the only mammalian species that can be easily manipulated genetically with well documented brain anatomy, physiology and function.
Moreover, many mouse models resemble various human diseases thus providing an excellent possibility to advance clinically relevant studies.

Most of our transgenic manipulations are not expected to cause discomfort, pain or distress to the mice. Moreover, our initial observations of the transgenic mouse strain we are working with indicate that these animals appear healthy and do not show any signs of discomfort. If unexpected adverse effects should develop (e.g., neurological signs such as tremor, circling behaviour, hyper-activity) the animals will be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will mainly focus on the practice guidance from NC3R in ensuring our experiments are conducted in the most refined way. Moreover, we will collaborate with scientists who are experts is specific procedures if necessary to obtain their advice and guidance. We will always be liaising with NVS and local BSU Named Persons to obtain guidance when necessary.

All researchers involved in the current project will follow the guidelines. "The Responsibility in the use of animals in bioscience research" general guidance document will be used as a reference document, as it sets out the expectations of the funding bodies for the use of animals in research.

The updated ARRIVE guidelines (2.0), designed for transparent reporting of animal research methods and findings, will be consulted for the planning and design of new experiments to ensure reliability and reproducibility of findings.

The “Guidelines for planning and conducting high-quality research and testing on animals” (2020, ref.
1) and PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines (2018, ref. 2),

.1. Smith AJ (2020) Guidelines for planning and conducting high-quality research and testing on animals Lab. Anim. Res. 36:21. doi: 10.1186/s42826-020-00054-0. eCollection 2020.

2. Smith AJ et al. (2018) PREPARE: guidelines for planning animal research and testing. Lab. Anim. 52(2):135-141. doi: 10.1177/0023677217724823.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will be informing myself by following NC3R guidelines and monitoring their news releases. Moreover, there are regularly symposia on new developments in NC3R which I will attend in order to further inform myself about advances in the latter. One of the goals is to gradually switch to using organoid

systems to address the questions we are investigating and avoid using animals. I will be pursuing this as a collaboration with experts in the use of such systems.

162. Pharmacology of Respiratory Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Respiratory disease, Inflammation, Efficacy, Infection, Treatment

Animal types	Life stages
Mice	adult
Rats	adult
Guinea pigs	adult
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

A review in the Lancet journal (2020) describes respiratory diseases as being amongst the most common worldwide, affecting in 2017, an estimated 545 million people (an increase of approximately 40% over the last 30 years). The aim of this project is to conduct experiments to facilitate the discovery and development of potential new treatments of respiratory disease by generating necessary pre-clinical data prior to the start of clinical trials in man.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

With an ever-increasing global burden, current therapies primarily provide only symptomatic relief or slowing of the disease while often exhibiting undesirable side effects. Consequently, there remains an urgent need to identify novel and more targeted treatments to combat these conditions. The work under this project licence will provide the necessary preclinical data in the development of such novel and/or targeted treatments in conditions such as asthma, chronic obstrucive pulmonary disease prior to clinical development.

What outputs do you think you will see at the end of this project?

The prmary output of this project will be to identify compounds/drug candidates including possible vaccines that have efficacy (both therapeutic and/or prophylactic) in animal models of respiratory disease so as to direct the progress of clients novel treatments into the clinic. Over the course of our current 5 year project licence we have tested in the region of 100 novel test agents in such disease models focused mainly on asthma, respiratory infection and acute respiratory distress syndrome (ARDS). Of these test agents I am aware that 10 have progressed into clinical development by our clients. I would therefore anticipate a similar or slightly increased volume of test agents being evaluated during the duration of this project licence with an increasing emphasis on respiratory infections.

Publications in peer-reviewed journals. We will also encourage clients to publish in peer-reviewed journals as much as possible so that data in readily available to the scientific community both in respect of agents that have shown good efficacy and those that have not, or indeed those that have highlighted safety concerns.

Who or what will benefit from these outputs, and how?

The primary benefit will be to patients living with chronic respiratory conditions such as asthma or idiopathic pulmonary fibrosis, acute respiratory conditions such as pulmonary infections and the associated complications that are associated with these conditions such as cough. The timescale for such benefits to reach patients following work conducted under this license based on current pharmaceutical development would be approximately 10 years, most of which would be time spent in clinical development. However, some test agents that have regulatory approval for other indications and being developed for other respiratory indications, will have already gone though some relevant clinical phase development previously which will allow a quicker (approximately 5 year) timescale for benefits to reach the patient.

The project may also expand scientific knowledge by identifying novel disease related mechanisms that help direct current and future research and testing of new therapies in more relevant disease models.

How will you look to maximise the outputs of this work?

Where possible and if required, with agreement of the clients, data we will published or encourage our clients to publish, in peer reviewed journals or present findings at relevant scientific meetings so that positive results can be dissemination amongst the scientific community or indeed negative results so that re-testing in animals of materials, particularly those that are unsafe, is avoided.

Species and numbers of animals expected to be used

- Mice: 6200
- Rats: 800
- Guinea pigs: 1500
- Rabbits: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult guinea pigs, mice, rabbits and rats are primarily the species used in pre-clinical research for respiratory disease as they have a fully developed nervous and immune system which underlie most airway diseases and conditions. These species are the most common that are used for research in this disease area and there is an abundance of scientific literature and global expertise demonstrating the rationale for their use. Indeed, the airways and general systemic physiology of these species, which contribute to the disease process as well as the pharmacokinetic profile and pharmacodynamic response to therapeutics is comparable to that seen in man.

Typically, what will be done to an animal used in your project?

In typical studies animals will receive agents to induce pulmonary inflammation that activates immune pathways linked with respiratory diseases and pulmonary infections. This will normally be induced by allergens (house dust mite), infections such as influenza or pseudo-infections using agents such as endotoxins from bacteria. These will typically be delivered either intranasally, intratracheally or as an aerosol to direct the inflammation topically to the lung. In some cases it may be necessary to initially sensitise the immune system when administering allergens so as to ensure the airways respond to the allergens when subsequently delivered directly to the airway. Typically systemic administration will be via intraperitoneal or subcutaneous administration. Typically, the induction of inflammation to induce the animals to be sensitised to the allergen and evoke an acute inflammation mirroring what is observed clinically in asthmatic phenotypes normally takes between 14 -28 days. In studies using pseudo-infection agents the acute immune response can be observed very acutely in comparison ranging from 3 hours to 24 hours following exposure, where the later time points develop more of an ARDS type response. In studies where infections are administered with typical respiratory infections such as influenza or coronavirus, a slightly longer study duration is typically used to ensure lung infection has developed and more representative of a clinical setting. For most of these viruses this tends to be between 3-5 days.

Typically during these studies test agents will be administered either as a single bolus dose or repeat dosing (usually daily) either therapeutically or prophylactically and will normally be delivered topically via intranasal, intratracheal or aerosol administration. However, some new therapeutics may be delivered systemically. Terminal samples will be collected from the animals to assess the impact of the test agents on the immune response and the resulting inflammation, this will primarily be by looking at cell numbers and biomarkers in alveolar lavage fluid and lung tissue. In some studies it may be relevant to look at the effect of test agents on airway function in animals that have been exposed to

allergens or infections/pseudo-infections, which following induction of pulmonary inflammation becomes hyper-responsive to normal neurotransmitter release. This will typically be assessed in animals that are under general anaesthetisia so as to allow the most detailed assessment of lung dynamics in a brief (30 min) protocol where an aerosol of synthesised neurotransmitter will be delivered. However, to look at the duration of effect of test agents at significantly improving lung function, studies in conscious animals will typically be required. Such studies will involve animals being exposed to the aerosols of synthesised neurotransmitters that will evoke a transient (1-2 min) mild bronchoconstriction.

Studies relating to lung fibrosis will typically involve the animal receiving a single administration of a fibrogenic agent such as bleomycin. This is normally delivered intranasally, intratracheally or oropharangeally so as to trigger the fibrosis specifically within the lung. Following administration of the fibrogenic agent, fibrosis progressively develops, which replicates what is seen in the clinic and culminates in a significant lung fibrosis by day 21 to 28. Studies will typically involve administration of test agents, usually by daily systemic administration i.e. orally over a number of days once fibrosis starts to progress, in a typical study this would be from day 14 (when initial inflammation has resolved and fibrosis has developed). Like with the allergen and infection studies described above, terminal samples i.e. lung tissue will be collected to assess the impact of test agents on the level of fibrosis developing in the lung.

In some studies it may be relevant to look at the effect of efficacious test agents on airway function in these animals. Fibrosis develops a progressive decline in lung function and this wiill be typically assessed in animals that are under brief (15-20 min) terminal general anaesthesia so as to allow the most detailed assessment of lung dynamic changes and any associated efficacious benefit offered by the test agent. Typically this will be achieved by assessing changes in dynamic compliance (a measure of the lungs elastic recoil during breathing) which will decrease in fibrosis.

Studies related to cough will typically involve conscious animals being exposed to aerosols of agents known to evoke cough such as capsaicin. This will usually be a 10 min exposure and cause a mild transient irritation of the airway to induce a cough reflex. In these studies test agents will normally be administered systemically i.e intraperitoneally or orally as a single therapeutic blous dose. However to look at chronic cough associated with respiratory disease such as fibrosis and asthma, typical cough experiments will be carried out in animals that have been exposed to allergens or fibrotic agents as detailed in the paragraphs above. These animals will then go thorugh the same typical aerosol exposures for 10 min to agents that evoke cough.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the animals are expected to tolerate well the acute exposure to stimuli for inducing inflammation, cough or fibrosis. In some animals (less the 1%) however, the induction of fibrosis may lead to weight loss and allergen exposure may cause transient localised inflammation around administration sites. Interventional steps will be taken to address stimuli and treatment effects that impact on animal welfare.

It is also excepted that animals will also tolerate drug treatments well in these disease models. On the rare (less than 1%) occasion when a drug treatment does impact on animal welfare, interventional steps will be implemented.

Expected severity categories and the proportion of animals in each category, per species.
What are the expected severities and the proportion of animals in each category (per animal type)?

We anticipate that animals will experience mild (75%) or moderate (25%) severity of pain/discomfort as a consequence of allergen/fibrogenic agent exposure and the resulting airway inflammation or fibrosis. We do not anticipate animals under this project will experience severe severity of pain/discomfort following any procedures.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are currently no in vitro models that involve a complete integration of the complexities of airway neuronal control and immune responses underlying many of the respiratory diseases/conditions. Nor are there in vitro alternatives which would completely model the complexities involved in absorption, distribution, metabolism, excretion or the safety of test agents. Indeed, there are currently no alternatives to in vivo testing that robustly replicate the complete complexity of whole organisms.
Consequently in vivo studies remain necessary in the search for safe novel therapeutic agents for respiratory diseases/conditions.

Which non-animal alternatives did you consider for use in this project?

Alternative approaches exist for looking at specific aspects of disease and the therapeutic potential of novel agents. This may involve 2D approaches i.e. single or co-culture of pertinent cell lines/primary cells such as lung fibroblasts and/or human epithelial cells, use of organoids, 3D air liquid interface (ALI) and ex-vivo models of isolated tissue or inflammatory cells. Indeed, we will look to explore the use of 3D ALI models to evaluate the efficacy of novel treatments.

Why were they not suitable?

In all cases, proposed studies will be carefully considered for alternative approaches in vitro and ex vivo first. However, using animals in vivo offers more predictive methods to assess the complex integrated processes controlling absorption, distribution and interaction of test substances and functional effects on neuronal and immune control relating to respiratory diseases. Where it is deemed appropriate to conducted an in vivo disease model study, all relevant/necessary ADME data will need to be obtained first by the client so as to ensure test agents achieve suitable systemic/topical exposure in the animal species.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers are based on our experience of study design based on usual client requests. Typical studies will include three treatment groups with test agents so that a efficacious profile of the test agent can be determined. Control groups that would normally be included would be a positive control normally in the form of a recognised clinical standard of care that we have previously acquired background data from. This would have two roles to play in ensuring the study provides maximum output 1) will directly compare test agents efficacy statistically to that of current standard of care thus helping with justification for go no-go decision making with the test agent. 2) demonstrate that the observed efficacy in a specific study correlates with expected results and that the observed effects of a novel test agent are valid for decision making. Negative controls will also routinely be included in a study design which will form the basis from which test agent efficacy is statistically analysed for biological effect.

Each animal will be maximised for data analysis where samples will be collected from all relevant tissues/fluids (matrices) to fully profile test agent efficacy in the disease progression i.e lung lobes, blood plasma, bronchoalveolar lavage fluid will be routinely collected. Although we will encourage clients from the outset to evaluate all matrices, it may be prudent for the client initially to focus on one or two matrices to gain an understanding of test agent efficacy, from which decisions can be made for additional analysis. Having taken all relevant matrices we will be able to provide the extra analysis without conducting further animal experimental work.

If the intended study disease model involves for example the use of a novel allergen or allergen sensitisation protocol or the proposed test treatment mechanism is completely novel we will look to progress with a small pilot study initially prior to progressing with a full statistically valid study as outlined NC3R's resource for conducting pilot studies.

Test treatments will be dosed blinded as much as possible. In addition studies will be conducted based on the principles outlined in the ARRIVE guidelines for randomisation with particular attention for nuisance variables.

Numbers of animals to include in studies are based on typical variations from our previous experimental work and statistical advise to calculate the minimum number of animals to be used whilst ensuring that the results are statistically significant.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of animals to be used is based on extensive experience of designing and running studies of this nature.

A power analysis will be conducted to determine the minimum sample size for each study.

The minimum number of animals used is also based on an understanding of the variation between animals, the sensitivity, and the reproducibility of the procedures to be undertaken. Statistical advice will be sought when required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies are frequently used when developing models and sharing of model information with collaborators to reduce the total number of animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The nature of the licence is that the most refined, relevant and least invasive techniques will be used at all stages. Therapeutic compound testing in these models will be with minimum burden to the animal's welfare i.e. minimum duration of study and stimulus required for experimental window to deliver on the required end point.

Surgical techniques and post operative recovery standards will be continuously monitored and revised to accommodate improvements. Such techniques will be conducted in line with best practice guidelines (LASA Guiding Principles for and undertaking Aseptic Surgery).

In more chronic studies, the dose route will be discussed with the client and alternatives to repeated injections such as implantation of minipumps will be considered.

We will be continuously monitoring the literature and will also involve the named animal care and welfare officer and named veterinary surgeon so as to implement the latest husbandry and environmental enrichment practices.

Substances administered will be in the smallest appropriate volume or lowest infusion rate for the shortest time practicable based around laboratory animal science association guidelines (Good Practice Guidelines - Administration of Substances) and relevant literature for substance administration
i.e. Administration of substances laboratory animals: Routes of administration and factors to consider, Turner et al (2011).

Why can’t you use animals that are less sentient?

The role of mice, guinea pigs, rats and rabbits are well documented and validated in the scientific literature and pharmaceutical industry for pre-clinical respiratory research depending on the pharmaceutical properties of the therapeutic agent, end point employment and knowledge of the target. Respiratory disease/conditions predominantly

have an underlying immune (either innate or adaptive) response that often leads to a change in a compromised pulmonary functionality through modified neuronal control of the airway. The species detailed in this licence biologically replicate the complex interactions of human immunity and neuronal control mechanisms that contrive to cause such disease/conditions in the lung. A process that is not so clearly observed in less sentient or immature life stages. In silico computer modelling also exists and is used by some companies to screen substance before going into animals. However, this in silico modelling fails to fully replicate the complete complexities of biological interplay between immune response and/or neuronal control mechanisms in the airway but does provide an approach to assess substance activity in relation to specific aspects of biological function.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We continually review our care procedures and each of the project license holders learn from each other with respect to procedures and refinements. Our named animal care and welfare office, named veterinary surgeon and named information officer attend regular national meetings and provide us with feedback on learnings.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Experiments will be planned so they can be reported in line with the NC3Rs ARRIVE guidelines. Regular literature reviews will be conducted to ensure our work aligns with best practice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Regular discussions will be held with the named animal care and welfare officer, named information officer and named veterinary surgeon in addition to receiving regular updates from the NC3R's. 3R's is also an agenda item during all our animal welfare ethical review body (AWERB) meetings which all establishment project licence holders attend and contribute too. For example discussed recently at our AWERB meeting under the 3R's agenda points following a member attending a NC3R's webinar, was the implementation of overnight fasting in studies and that this practice may well offer no significant scientific advantages and indeed is more likely to have a negative scientific impact.

163. Genetic Manipulation of Rodent Plasmodium spp.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Malaria, Transmission, Sexual development, Antimalarial drug discovery

Animal types	Life stages
Mice	juvenile, adult
Rats	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to improve the fundamental understanding of the biology of Plasmodium, the parasite that causes malaria, using rodent infecting Plasmodium species as a model for further research into the human disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In 2010 the World Health Organisation (WHO) reported an estimated 216 million cases and 665,000 deaths from malaria. Worryingly, following two decades of decline in both case numbers and mortality rates, in the last year both metrics have increased (241 million and 627,000 in 2021 on WHO website) and emphasises the persistent threat that malaria causes. Control continues to be attempted predominantly through distribution of medicines

and insecticide-impregnated mosquito nets. However, we cannot rely on the use of antimalarial medicines for long-term prevention and treatment of the disease. Resistance to drugs such as chloroquine and other quinine related compounds continues to spread. In addition toxicity problems have arisen in other prescribed medicines such as Mefloquine, (Lariam) and resistance to the current front line treatment Artemisinin is widespread in SE Asia.

Therefore in order for this disease to be controlled and hopefully one day eradicated, it is a valid argument that we must first understand the biology of the parasites that cause malaria, their interactions with their hosts and with the mosquito vectors that spread malaria.

What outputs do you think you will see at the end of this project?

The principal benefit will be increased fundamental knowledge of the biology of the malaria parasite through all stages of its life cycle and from this, to identify points we can target to prevent completion of the life cycle. There is one stage where this may be more possible than others, and that is the transmission stage – the point at which the parasite can transfer from host (eg human) to vector (mosquito) and back into a host (eg human). The World Health Organisation proposes that blocking this transmission is one of the most effective approaches to the control of the disease. Our work should provide further experimental support for this proposition.

We also provide support for our immediate research community through the provision of data and reagents, protocols and specialised parasite lines that can be used by other research groups. Any results will also be published in peer reviewed journals. Within the greater global research field, our research may further the discovery of potential vaccines against malaria which can then be tested in the model we use prior to further development. In a similar spirit, drugs and antimalarial compounds might be subjected to animal testing before their use in clinical trials.

Who or what will benefit from these outputs, and how?

We study the rodent infecting malaria parasite, Plasmodium berghei, as it gives access to all life cycle stages (in mammalian liver and blood, and in mosquito midgut and salivary glands), which cannot be achieved in the study of the human species. Our research will focus on disruption or modification of DNA / RNA / protein known to, or expected to, control the sexual stages of the parasite life cycle. It is the sexual stages which become transmissible (ie transfer from mammalian host to insect vector then back into another mammalian host via mosquito bites) continuing the spread of the disease. As malaria kills more than 600,000 people a year, is once again resurgent, and resistance to current medicines is so widespread, a long-term programme of control is desperately needed. Effective control of transmission is one of the most effective ways to limit the spread of the disease.

Another potential benefit of our research could be the identification of vaccine targets which would block transmission. Our system will be used to test potential vaccines, or other drugs and antimalarial medicines, before their use in clinical trials. These are longer term aims. In the mid-term, the malaria research community will hopefully benefit from any results that we generate, further aiding the research and outcomes from other groups.

How will you look to maximise the outputs of this work?

We are an extremely collaborative research group housed within one of the foremost parasitological research centres in the world. Our overall ethos of our collective is one of data and research sharing ideally before publication even at the preprint stage. We have shared parasite lines, protocols and large datasets (in the well-known, publicly accessible servers and websites e.g. VEUPATHDB.org) as a routine part of our work pattern. We have participated in and organised training courses to further disseminate our skills and techniques. We will continue to publish our work in peer-reviewed journals and make full use of pre-print servers. We have collaborators in the USA, Malaysia, Sweden and across the UK.

Species and numbers of animals expected to be used

- Mice: 3000
- Rats: 50

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use mice and rats as our research is focused on the rodent malaria parasite Plasmodium berghei as a model system for human malaria. Unfortunately it is not possible to grow the parasite through its entire life cycle outside of the animal in the lab, and therefore we need to utilise mice and rats as a host in order to access all stages of the parasites development.

Typically, what will be done to an animal used in your project?

Animals will be infected with malaria parasites by injection, either into the abdomen, or into the tail vein. Approximately 3 days following injection parasite levels are assessed once per day by looking at a small amount of the animals' blood (which is obtained by pinpricking the animals' tail) under a microscope. In most instances the parasitemia (number of parasites in the blood) is at the required level between 3 and 7 days after injection. Infections are not permitted to get high enough to cause illness in the animal. If any animal appears unwell, they are euthanized immediately. Once parasites are detected in the blood, and reach a desirable level, the animal is heavily sedated, the blood removed, and the animal humanely killed. If parasites are not detected within 7 days, the animal will be kept for use in one further procedure.

Procedures that would cause pain are performed under sedation. This includes the procedure to test whether the parasites are transmitted from an infected animal to mosquito or from mosquito to uninfected animal via a blood meal. In this instance the animals are under general anaesthesia and are placed on top of a mesh mosquito cage, their paws, tail and face protected from the insects. The mosquitoes are allowed to feed on two mice for up to 15 minutes. The mice then recover and are assessed on a daily basis for infection. The protocols are designed to involve minimal stress to the animals.

What are the expected impacts and/or adverse effects for the animals during your project?

Out with the discomfort of injection, the degree of suffering to the animal is minimal. All protocols are designed to involve minimal stress to the animals. Most routinely used protocols require no or minimal anaesthesia. Any procedures that would cause pain are performed under sedation. Also, we do not allow infection to develop to the stage of physical symptoms of rodent malaria eg anaemia, weight loss, laboured breathing. Our protocols are further designed to incorporate administration of drugs via drinking water, rather than injection, wherever possible, and the minimum numbers of mosquitoes are used for mosquito feeds. Furthermore, we use mice that are not susceptible to experimental cerebral malaria (ECM), a condition that can result in convulsions, coma, and death and so our mice should not develop these symptoms.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Procedures performed on the animals are of either Mild (95%) or Moderate (5%) severity, with the blood maintenance feed being non-recovery.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The rodent malaria model allows access to all stages of the malaria parasites lifecycle for research. However, it is not possible to grow Plasmodium long term in culture in the lab. Access to all stages of the life cycle requires the use of rodents as a host for the parasite, and mosquitoes as the vector. This comprehensive coverage of the parasite life cycle is not available for research in human species of Plasmodium.

Which non-animal alternatives did you consider for use in this project?

We can, and do, grow the malaria parasite in culture in the lab. This is performed routinely to allow experimental finesse such as synchronizing the life stage of the parasites or for larger scale testing of drug sensitivity for which we have generated extensive protocols. However, growing parasites outside of the animal only works for specific stages of the parasite’s life cycle, and they can only complete one cycle.

Why were they not suitable?

If we want to study the entire life cycle of the malaria parasite then we have to use animals. Unfortunately there is no alternative as the parasites do not complete their life

cycle in culture in the lab. We can mature parasites in the lab to complete a single cycle but no further. In order to study all of the life cycle stages, and specifically the important transmission stage between host and vector then we need to use animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have on average 5 lab members working on the rodent malaria model. We will use approximately 500 mice, and 10 rats per year. We require animals to grow and replenish our stocks of modified

parasites, in order that we can continue to study the genetic modifications. The rats are predominantly used to expand our existing parasite lines because we can achieve a greater parasite bulk from a single rat as opposed to the use of multiple mice. Although new methods to isolate specific populations of genetically modified parasites are being reported, these methods are not routinely used as yet. The most common method still requires 10 mice for every modified parasite. As the newer methods become more established we will utilise them, and reduce our animal numbers. In order to state experimental results with assurance we also need to ensure our data has statistical significance. This therefore requires experiments to be repeated. Approximately 100 mice/year are also used to maintain our mosquito colony.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use animals to generate mutant parasites to study the role of proteins on Plasmodium parasite biology. Our reduction in animal use is paramount, generated through refinement in methods and since the start of our investigations into Plasmodium berghei we operate a continuous cycle of procedural improvement. For example, we have modified methods so that rather than using 50 mice to generate one mutant parasite, we now only need between 5 and 15 mice depending on the experiment. We have also developed a mutant parasite line whose life cycle can be controlled to generate a specific life cycle stage, and rather than needing 20 mice to obtain enough parasites for our experiments we now only need one mouse to obtain ample parasite numbers. Thus, in the last 5 years my programme of work that required nearly 10,000 animals is now forecast to require 3000 for the forthcoming 5 years.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Experiments are planned very carefully to ensure that the minimum number of animals are used, and whenever possible animals will be shared between lab members. A number of methods have been refined to decrease animal usage. For example, a method by which we select a specific parasite population in an infected mouse now requires the use of one mouse rather than the four mice required previously. Likewise in certain cases we can

collect specific parasites by a method called FACS that uses one mouse, rather than ten mice required with another method. We also use a strain of mouse from which we can obtain a greater blood volume in comparison to other strains.

If an animal does not become infected with the parasite following injection, then this animal can be used for one more another attempt at infection, further reducing the number of animals required.

Plasmodium readily infects both mice and rats. Rats are used infrequently when a large yield of parasites from a single host is required in order to minimise mouse numbers and potential variability.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare

costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use a mouse model to study malaria as it allows access to all stages of the life cycle of the parasite. In other research groups, mice are typically infected with Plasmodium to study a phenomenon known as Experimental Cerebral Malaria (ECM) whereby the parasite infects the brain and the animal can suffer convulsions, coma and death, which is thought to be analogous to cerebral malaria experienced by many people infected with the human malaria parasites. Some strains of mice infected with the Plasmodium will develop ECM at day 9-10 after infection and die after convulsions unless treated or euthanized. However, since ECM is not a topic of our research we avoid the use of mouse strains that are susceptible to it.

Animals are injected with parasites and monitored daily by taking a very small amount of blood by pricking their tail and looking at it under a microscope to determine parasite numbers. If an animal has a low number of parasites, but looks as if it is ill and suffering (eg hunched over, scruffy fur, hiding in the corner of the cage, pale ears/feet) we humanely kill the animal.

Why can’t you use animals that are less sentient?

Plasmodium only infects mammals, birds and reptiles, and each species of Plasmodium has a very restricted host range to which it is infective. Plasmodium berghei is a rodent malaria parasite and closely resembles the human parasite life cycle. P.berghei will only grow in rodents. This model has been developed over the last 25 years to be one of the most sophisticated and genetically amenable allowing fundamental, novel insights into the general biology of the parasite.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Recent advances in the identification of a gene which controls the production of a specific stage in the parasite life cycle - the gametocyte - has enabled our group to refine our methods, decreasing the chance of harm to the animals. Previously we had to allow a much higher infection rate in the animal to obtain high numbers of gametocytes. Research is continuing into this gene to ensure that the gametocytes produced are like those found naturally. If this is the case, then significantly fewer animals will need to be used for gametocyte studies in the future.

Alternative methods of drug treatment have and will continue to be researched. If drugs can be administered orally via drinking water and similar results achieved as with injection into the abdomen or the tail vein, then procedures will be modified to incorporate these. We have successfully implemented such procedures for the two main drugs used to select for genetically modified parasites resulting in greatly reduced animal handling and exposure to scheduled procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We monitor the scientific literature including pre-print servers in order to stay abreast of developments in our field of study and to become aware of improved practice with P.berghei. We may also learn of improvements through participation in scientific meetings and workshops. For example recently we have refined our use of a method to modify DNA - the genome editing tool, Crispr/CAS-9 - in the laboratory as a result of discussions with and receipt of tools from innovators in China.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our Biological Services unit arranges bi-monthly meetings for Personal Licence holders where we are informed of recent advances in the 3R's. Group members also attend the annual 3Rs day and Culture of Care meetings. Biological services also forward relevant emails / correspondence from the NC3Rs.

164. Effects of Nutritional Adequacy on the Health and Wellbeing Across the Lifespan: From Conception to Old Age.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

maternal, fetal, metabolic diseases, lifespan, alteration of nutrition

Animal types	Life stages
Mice	pregnant, neonate, adult, juvenile, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To assess how poor nutrition during pregnancy or early infant life influences the health and wellbeing of the mother, developing foetus and early infant.

To identify and potential diet or drug interventions during postnatal life that can prevent the risk of developing non-communicable disease in adult life.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
It is well established that exposure to poor nutrition during foetal development and or early postnatal life increase the risk of developing non-communicable diseases in adult life such as metabolic diseases (high blood pressure, obesity, diabetes, cardiovascular disease) and cognitive diseases (Parkinson's, Alzheimer's disease) and evidence suggests the same conditions even extend to subsequent generations. This area of research known as 'developmental origins of disease', is where insufficient or excess nutrition during critical windows of early development cause permanent changes to key cell types within tissues and organs and the physiological metabolic function in the foetus and early infant that increase the risk of non-communicable disease in later life. The NHS is overwhelmed with having to treat people with long-term non-communicable medical conditions. In the UK, 3.9 million individuals have type II diabetes, and 58% of women and 68% of men are obese or overweight (National statistics, 2017). Metabolic diseases are also associated with cognitive deficits with 690,000 people in the UK now suffering from dementia. The economic costs to the NHS are rapidly increasing and appropriate interventions are becoming crucial. It is predicted, that the cost of treating non- communicable medical disorders will annually exceed £49 billion. As a result it is has become critically important to understand the mechanisms whereby maternal nutrition increases disease risk to both the mother and her offspring and to identify potential diet or drug therapies to improve health outcomes vital to help the NHS.

What outputs do you think you will see at the end of this project?

The proposed research programme will use established animal models of nutritional deficit or excess during foetal or early infant life to 1) uncover the important molecular, physiological and cognitive

mechanisms associated with metabolic and cognitive disease in the adult offspring and 2) to identify appropriate postnatal windows for dietary or pharmacological intervention.

Key but not exclusive outputs from the study will include the effects of maternal over or undernutrition on 1) body composition and metabolism (bodyweight, food intake, glycaemia, musculoskeletal strength, and flexibility), 2) effects on cognitive behaviour (anxiety, memory and learning) and, 3) expression of key proteins in tissues (adipose, muscle, brain) associated with body composition and metabolism and cognitive behaviour.

All data will lead to publications in leading scientific journals, articles in the popular press and presentations at both UK and international conferences attended by experts in the field from around the globe.

Who or what will benefit from these outputs, and how?

This research aims to understand how maternal nutrition during foetal and early development impacts on biological systems to promote health as well as identifying a strategy to improve lifelong health and wellbeing. The immediate to short-term impact of the work will be accumulation of new scientific knowledge for basic research scientists with an interest in nutritional biochemistry, metabolic regulation and musculoskeletal health. Long-term impact of the research will benefit health care professionals, involved with obstetrics and gynaecology, maternity and childcare services (clinicians, midwives, health visitors) where the projects findings can be used to provide dietary advice to pregnant women and new mothers. The data generated from the project will also help to inform future public health and clinical policies relating to dietary and pharmacological

interventions and the impact of the proposed research will extend beyond the timeframe of the project licence.

How will you look to maximise the outputs of this work?

The new knowledge resulting from the research will be publicised by presenting the data at local, national and international scientific meetings (e.g. Nutrition Society, British Society for Neuroendocrinology, Society for Endocrinology, Society for Reproduction and Fertility, Physiological Society, Association for the Study of Obesity, World Congress on Developmental Origins of Health and Disease and the Endocrine Society). Project findings will also be published in open access high impact journals such as FASEB, Clinical Science or PNAS.

The proposed work will lead to internal collaborations within our research facility, to investigate the impact of vitamin D deficiency on epigenetic markers), bone health and maternal health during pregnancy as well as International collaborations by investigating the impact of nutrient deficiency or excess on development of neurodegenerative diseases at Universities we have links to in North America and Italy. .

Species and numbers of animals expected to be used

- Mice: 2800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The proposed research will often utilise rodent models as they have already been extensively characterised and remain the most appropriate models through which to address our research questions. The key advantages of using rodent models are that they exhibit 1) short pregnancies and life-span, ideal for reproductive studies 2) robust reproductive performance; 3) ease of dietary manipulation; 4) good availability of tools to investigate key proteins and physiological function of the whole animal (in vivo). In addition we have already characterised and established a rodent model of maternal obesity and vitamin D deficiency that mimic outcomes observed in the human population. Fundamentally having such well-characterised models will enable us to identify the how nutrition affects health and identify possible dietary or drug interventions that we can then test very easily.

Rodents will be assessed at different times of life from fetus to old age to mimic human lifespan, thus enabling us to understand how dietary changes during fetal development lead to lifelong disease and to determine whether supplementation or consumption of a control diet can prevent diseases progression or whether the development of disease is inevitable due to the exposure during fetal development. Furthermore, we are also interested in the health of the pregnant mothers as healthy mothers lead to healthy offspring.

For these reasons, rodents provide the most appropriate non-human model for such studies into the effects of parental diet on fetal growth and offspring health across the lifespan.

Typically, what will be done to an animal used in your project?

Under this project we will define how diet affects maternal and offspring health from the fetus to old age. As such two groups of animals will be studied, the parents and the offspring.

Typically, virgin female rodents will be fed ad libitum (as much as they like) of either a control or an experimental diet (e.g. vitamin D deficient, high in fat, high cholesterol high fat diet) either prior to mating (typical 6-12 weeks before) only, during pregnancy only, during lactation only or during all three stages. This change in diet is not expected to cause distress but may lead to obesity as observed in the human population. Some diets may result in weight loss due to unpalatability. In this instance, if acute loss of 10% or loss greater than 10% which hasn't started to recover in 72 hours occurs the animals will be placed on a control diet.

Bodyweight and food intake will be monitored at least twice a week, and further metabolic testing (glucose tolerance tests, blood pressure and assessment of energy expenditure (activity, oxygen consumption and carbon dioxide production (VCO2), ingestive behaviour (food intake, duration of feeding, frequency of meals), and physical activity) will be typically these assessments will be prior to pregnancy, and during pregnancy (0 and 16.5 days) and/or postnatal days 1 and 21. This will allow us to assess whether nutritional deficits/excess leads to gestational diabetes, pre-eclampsia, and excessive weight gain in the mother. At the end the dams (pregnant rodents) and offspring will be euthanized via schedule 1 method or via exsanguination (blood withdrawal from the heart under

terminal anaesthesia) either during pregnancy, labour or early postnatal age to assess changes in key proteins associated with gestational diabetes, pre-eclampsia, and energy expenditure (objective 1)

Additionally offspring could be transferred to another protocol following weaning to assess the effects of poor nutrition during development on lifelong health. Typically offspring may remain on parental diet or be switched to a control diet or be provided an alternative diet (e.g. low fat or high fat diet).
Bodyweight and food intake will be monitored at least twice a week. Metabolic assessment may occur at various times across the lifespan (e.g. weaning, 3, 9, 15 and 21 months) and the same animal maybe tested at each time point to assess the influence of age and diet or animals may be culled for hormonal and gene changes at any time point during the study via schedule 1 methods and/or exsanguination (objective 2).

Where possible each animal will act as its own control and will be tested at each time point to reduce the number of animals.

Metabolic assessment during this project will include

- Glucose and insulin tolerance tests – animals may feel some discomfort due to overnight fasting (maximum of 12 hours) although water will be provided at all times. Glucose or insulin will be administered via the abdomen or orally using small flexible catheter and blood samples will be obtained by either tail or femoral (saphenous) vein. Some monetary discomfort may be experienced following administration of substance or following blood withdrawal. The route of sampling will be dependent on whether two or more samples are required. If the saphenous (femoral) vein is used animal may be restrained manually or using a restraint tube which is typically used for conventional

sampling. Due to the smaller volume, it is generally quicker to collect micro-samples. Animals will be habituated to the tubes for one minute daily one week prior to sampling. Each animal will have the same tube during sampling to prevent stress or distress. During pregnancy only two samples (0 and 15 minutes) will be obtained to prevent unnecessary stress, while in the offspring this may be conducted up to 5 times (e.g. 0,15, 30, 60, 120mins).

- Blood pressure: will be determined using a Tail-cuff, similar to human blood pressure measurements. Typically, rodents will be warmed to 27◦C for at least 2 h before testing, and all animals will be handled and measured by the same technique and operator. The cuff will be inflated twice before measurement collection to acclimatise the animal to the procedure. Some transient discomfort is expected but this will not cause lasting harm.

- Energy metabolism: will be assessed using metabolic cages which allow the measure of ingestive behaviour (food intake, frequency of meals and duration of meals), oxygen consumption, carbon dioxide production and activity every 8 minutes. Typically animals are placed in sealed, temperature controlled experimental cages with access to as much food and water they may want, food maybe ground or whole pellets. As all measurements are performed in cages equivalent to the animals' home cage there should be minimum stress to the animals, however the cages do not contain bedding or chew toys (this interrupt gas flow measurements and activity monitors) and therefore can cause slight distress to the animals hence the requirement for 24-hour habituation. This duration of stress will be minimised by limiting time in the metabolic cages to the minimum required to obtain the required data. Typically, animals will be placed in the metabolic cages for minimum of 48 hours. The equipment is not gas tight so the animals will not asphyxiate if the power fails. Animals will be checked on daily and if any abnormal behaviour is observed animals will removed and returned to their home cage.

- Body composition (percentage fat to assess obesity), this will be conducted using X-ray computed tomography (CT) scanning. CT imaging will be carried out under gaseous anaesthesia, no adverse effects are expected although animals will be monitored for 24 hours to signs of food intake. This will only be conducted in offspring or virgin animals.

- Bone density and growth rate using X-ray computed tomography (CT) scanning and a method we have recently developed involving administration of fluorescent Red and green dyes at several time points with 5 day intervals until 3 months of age. No bone growth occurs following 3 months. The animals may feel some discomfort following the administration of substances and recovery from anaesthesia, however animals will be monitored for food intake for 24 hours. This will be conducted in offspring or virgin animals.

- Muscle strength: Grip strength will be assessed using a grip strength meter which is a simple non- invasive method designed to take advantage of the animal's tendency to grasp a horizontal metal bar or grid while suspended by its tail. Animals may experience some discomfort during the process as however this should not last and animals will be monitored for food intake and normal behaviour for 24 hours. This will only be conducted in offspring or virgin animals.

- Learning and memory, anxiety and stress will be assessed using validated tools such as novel object recognition, spontaneous alternation test (T-maze) and Y maze. These behavioural tests utilised are widely within the University and have been refined to give optimum results.

- Novel object recognition (NOR) takes advantage of the fact the rodent prefer to explore novel objects, shapes and locations, so first rodents are presented with identical objects and then one is moved to a new location or replaced by another. It involves three 10-minute trials separated by a 1- hour interval

- The spontaneous alternation test (T-maze) and Y maze: This is based on the willingness of rodents to explore new environments therefore prover to visit a new arm of the maze rather than a familiar one. In a free choice procedure of this test, animals are allowed to choose one goal arm during a first exposure. They will be confined to this arm for 30 seconds and a second choice will be given.
Animals will be tested ten times a day on three consecutive days.

- Open arena: Rodents tend to avoid open areas and will typically spend a significantly greater amount of time exploring the periphery of the arena, usually in contact with the walls, than the unprotected centre area. Rodents that spend significantly more time exploring the unprotected centre area demonstrate reduction in anxiety. Rodents are typically exposed for 5 minutes and anxiety related behaviours such as grooming will be noticed.

What are the expected impacts and/or adverse effects for the animals during your project?

Variation in diet: Animals will be fed experimental diets (typically vitamin D deficient, high fat high cholesterol or and high fat diet) and may experience significant changes such as gains in weight and changes in blood pressure and glucose/insulin levels. It is anticipated that these changes will last for the duration of the feeding regimen. However, these effects are not expected to result in significant pain, suffering or distress to the animals or their wellbeing or general condition, as observed in people consuming these diets. Some diets may result in weight loss due to unpalatability. In this instance, if acute loss of 10% or loss greater than 10% which hasn't started to recover in 72 hours occurs the animals will be placed on a control diet,

Substance administration: Animals will receive relevant substances (e.g. glucose, insulin, tracers for CT) via appropriate routes (mouth, by infusion into the blood via the tail, into the abdomen or under the skin). This will typically be a single injection and all routes are expected to cause momentary discomfort however in less than 1% there is a potential to lead to inflammation at injection site or lead to administration into the windpipe rather than stomach following oral administration. Breathing in animals will be continually monitored and only trained personnel will conduct administration. Any change in behaviour such as failure to consume food, looking hunched/shivering (piloerection) or having difficulty in breathing will result in euthanasia using an appropriate schedule 1 method.

Bleed from tail vein or saphenous (femoral) vein: Animals bled via these veins may temporarily favour the opposite limb and have mild bruising. However we do not expect this to last any longer than 24 hours, if this continues we will have a discussion with the staff and the named veterinary staff to ascertain what needs to be done. However competent individuals will perform the technique, no more than two blood samples will be taken from tail vein in any one 24 hours; no more than four blood samples will be taken from the saphenous vein within any 24-hour period. Small pressure will be applied to the sampling site to stop bleeding and animals will not be returned to their home cages until bleeding has stopped.

Metabolic cages: Some animals may be placed in metabolic cages, however as these are similar to home cages we do not envisage any lasting harm. Mild distress may be caused due to lack of enrichment (bedding / chew toys) as these interfere with gaseous exchange. Animals will be in these cages for a minimum of 48 hours. Food intake will be monitored for the duration of the assessment.

Blood pressure measurement: Some animals may have their blood pressure measured. Animals will be lightly restrained for the duration of the blood pressure procedure and may suffer mild, transient discomfort. This is unlikely to cause any pain or lasting harm. During the procedure the animals will be housed at a temperature above the normal housing temperature. This is not expected to cause any adverse effects, but animals will be monitored for evidence of heat shock and distress throughout the procedure – similar to individuals developing heat stroke.

PET/CT: No adverse effects are anticipated during the administration of substances for PET/CT, although mild nausea/diarrhoea (manifested as general ill appearance) and inflammation may occur following administration of tracers in less than 1%. Animals will be continually monitored throughout the procedure. At the end of the study if an animal is to be reassessed then they will be provided with a palatable diet (e.g. diet soaked in water) and food intake/bodyweight will be monitored a numerous times in the first 24 hours. Signs of infection will be treated with antibiotics or topical agent following discussions with NVS. If animals have undergone imaging, then animals may NOT undergo glucose/insulin tolerance tests.

Induction of anaesthesia: Potential for inappropriate anaesthetic depth that could result in pain or death (<1%) but this will be minimised by controlling and monitoring anaesthetic concentrations by watching any pedal reflex, corneal response and depth of breathing, this is a similar . Furthermore to prevent any drop in body temperature, as observed in rodents and humans following anaesthesia, warming equipment will be used. If the animal is to recover following anaesthesia animals will be monitored over the first 24 hour period which is critical. Weight loss will limited to a maximum of 10% or signs of illness (piloerrection, hunched in corner, increased vocalisation) will result in euthanasia by appropriate humane method.

Muscle strength: As mice are required to be pulled from the tail this may induce aversion and high anxiety levels. This will be performed by trained staff, animals will be monitored for 72 hours any signs of illness (piloerrection, hunched in corner, increased vocalisation) and/or weight loss of 10% within 72 hours will result in euthanasia by appropriate humane method.

Withdrawal of food: Weight loss may occur due to changes in availability of diet, for example food withdrawal prior to glucose or insulin tolerance tests. Typically, these will be overnight (maximum of 12 hours) with a minimum of 2 weeks between fasts. Water will be provided at all times. Weight loss is limited to a maximum of 10% in 72 hours. Animals will be weighed daily following procedure for 72 hours and then at least weekly. If weight gain is not observed within 72 hours, then animals will be euthanized by appropriate humane method.

Cognitive assessment (Neurodevelopment): Mild stress may occur due to removal from home cage and placement into an open arena, animals may fall off the maze. Animals will be continually monitored during the tasks for signs of distress, animals will undergo training prior to trials in the mazes and soft landing will be provided but if this occurs more

than twice then the task will be terminated. For any task excessive vocalisation or defaecation will lead to animal being removed and placed back into home cage. Animals will be monitored for 72 hours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Over the duration of the project it is anticipated that 100% of the animals will experience procedures that are of a mild severity limit, however as some steps maybe repeated over time this may lead to moderate severity. The advantage of repeating measurements over a lifespan enables the animal to be its own control thus reducing the number of animals alongside reducing variability.

As the majority of the animals will be consuming a diet varying in constituents this may lead to changes in physiology (e.g. weight gain, changes in hormones and pregnancy). However, in our experience none of these have caused mortality or the need to cull any animals due to adverse effects.

Further discomfort may come if the animals

- are placed in the metabolic cages (25%) due to lack of enrichment or if tested for memory and learning (25%) due to possible anxiety and stress. However, the effects of this will be temporary and are not anticipated to result in impairment of the well-being or general condition of the animals.

- tested for glucose/insulin (25%). These will typically result in mild severity over a short-duration. Effects will typically be in the form of soreness and swelling at the site of injection and are likely to occur in all animals receiving injections and are of a mild severity.

- anaesthetised using general anaesthesia (25%). This will typically result in mild severity over a short duration, animals will be monitored for 24 hours post recovery and offered palatable diets.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animal models to understand how diet influences maternal and offspring health across the lifespan, which is easier in rodents due to the short pregnancies and lifespan (e.g. 22 months in rodents = 60 years of age in humans).While we could conduct observational studies in humans, it would be unethical to manipulate the diet or not to

mention any observation that may be detrimental to the mother or offspring. Furthermore, the processes we would like to investigate such as food intake, bodyweight, development of diabetes, physical activity are complex that currently no in vitro system is able to replicate this.

Which non-animal alternatives did you consider for use in this project?

Prior to using animal models we try to test some hypotheses using cell (in vitro) models and have extensive experience in manipulating an number of cell types (muscle, brain, pancreatic, fat cells). An example of this is when we showed that very low physiological levels of vitamin D (equivalent to a deficient state) increased the formation of fat droplets in muscle cells if grown in fat (adipogenic) environment. This was associated with an increase in the expression of genes associated with the formation of fat cells, while higher levels (equivalent to sufficient state) inhibited all these effects. We were the first to show that VD could regulate the conversion of muscle cells into fat cells known as transdifferentiation. Similarly, VD has been shown to inhibit fat cells synthesis in the fat cell line).

These studies have led us to the hypothesis that possible maternal vitamin D deficiency may favour fat cell synthesis over muscle cell synthesis during development, thus leading to increased risk of obesity and diabetes in addition to the well-established observations (e.g. poor physical performance, increased risk of falls, and impaired muscle strength). One of the hypotheses we will be assessing in this protocol using animal models.

Why were they not suitable?

As we want to understand what the impact of nutrition on changes in key proteins and relate them to changes in physiology (bodyweight, memory learning, muscle strength etc.) across the lifespan, unfortunately cells are not suitable for this. We have considered the use of lower organisms and mathematical model systems, but these do not allow us to model the complex interactions between the various factors across the life course.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

All work is in accordance with ARRIVE guidelines. In order to ensure that high quality, reliable and valid data is produced from the minimum number of experiments, the ARRIVE guidelines (Kilkenny et al., 2010) will be followed when reporting the results obtained from this project. http://www.nc3rs.org.uk/page.asp?id=1357. This ensures that the data can be fully evaluated and utilised. Based on these measures to ensure that the minimum number of animals is used, an estimated number of animals to be used over the duration of the project. This is derived from the typical experiment representing a standard experimental design used in our laboratory.

Furthermore, numbers were estimated using the following calculation from the IACUC

To investigate the effects of diet on offspring health across the lifespan, requires a minimum of 2 parental groups (control and treatment) to produce approximately 4 offspring per litter (2 male and 2 female (sex correction)), although this may vary naturally and some litters may be lost during station or at birth would need replacing. Offspring will be assessed at five different time points across the lifespan, thus for the generation of 1600 offspring we would need 200 adult females per treatment group with approximately. In favourable times, we envisage at least two mouse experiments therefore we envisage the need for 800 breeders rodents and 400 stud males for protocol one leading to 1600 offspring in protocol 2; equating to 600 rodents per year. These numbers have been identified from pilot studies and previous published studies conducted by the group following the help of a statistician.

Furthermore, to maximise the data output from each animal where possible animals will act as their own controls to reduce the number of animals and variability across the lifespan. Alongside this will take utilise all tissues of the animals by collaborating with other groups who may be interested in other diseases (e.g. vitamin D deficiency and lung performance).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used the NC3Rs experimental Design Assistant to confirm that the number of animals mentioned above. We will use the EDA diagram and report outputs to support experimental planning with animal users.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Apart from good experimental design we will conduct the following to optimise the number of animals utilised.

Use of pilot data: this is a useful part of the overall research strategy to test logistics and gather information prior to a larger study. This provides vital information about the number of animals needed and how we can reduce the numbers as well as making sure the design answers the research question.

Efficient breeding: We will choose animal strains which are known to breed efficiently relying on strains the group have previously used. We will use a study design for mating such that animals will be mated in batches following the ‘worked example of breeding’ from the NC3Rs website (Worked example of intermittent breeding | NC3Rs), for example 12 females would be mated with 6 males to produce 48 pups (see figure below). Males will be removed when pregnancy is confirmed. Offspring are weaned and genotyped.

Use of in vitro (cell) models - where possible we will use alternatives to animals to test our hypothesis first and our team has an excellent record of accomplishment using these models to make sure any animal work conducted is hypothesis driven thus reducing the number of animals required.

The use of gold standard techniques to minimise variation: One of the key outcomes of our research group is that we are experienced in the use gold standard methods to minimise distress of the animals. Furthermore we will continue to search the literature for new

instrumentation or innovative. For examples we use metabolic cages so we are able to get more multiple measurements

The use of

- metabolic cages to assess energy expenditure, appetitive behaviour (intake, meal numbers) and physical activity over a period of time which reduces the number of animals required for experimental procedures and improves the quality of the data as multiple measures are recorded simultaneously from the same animals.

- In vivo imaging such as positron emission tomography (PET) to assess body composition and glucose uptake can reduce animal numbers by almost 60-70% and replaces traditional immunohistochemistry (where the tissue is required prior to analysis).

Literature searching: Continue to read the literature to determine any new Instrumentation and Techniques. Using new instrumentation or innovative techniques that can improve precision can reduce the number of animals needed for a study. Indeed using PET/CT and the TSE system will reduce animal numbers while increasing the data obtained from each animal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Why these models:

The studies will use rodent models as the key advantages of using rodent models are that they exhibit
1) short gestation lengths and life-span, ideal for reproductive studies 2) robust reproductive performance; 3) ease of dietary manipulation; 4) good availability of tools to investigate gene expression and physiological function. In addition to the advantages described above, we have already characterised and established a translational rodent model of maternal vitamin D deficiency and obesity. Fundamentally having such well-characterised models will enable us to identify the mechanisms involved and possible dietary or drug interventions that we can then test easily. Many of the studies we are conducted are similar to those already conducted in humans.

In addition we will use methods that reduce pain, suffering, distress, or lasting harm to the animals such as using flexible catheters for oral administration over traditional stainless steel rigid ones – these are less likely to cause trauma and dosing in the lung. These are similar to those used in humans during intubation or use of tail or saphenous (femoral) vein bleeding rather than surgical implantation of a catheter and immobilisation. This allows animals to be conscious and free thus reducing stress, again this is something conducted in humans.

Why can’t you use animals that are less sentient?

As stated previously we have a strong record of accomplishment using in vitro (cell) models, however these would not allow us to display dramatic differences in physiology and gene expression. White non-mammalian models do not share significant similarities with humans such as having placentas or uterus and therefore we would not be able to assess how diet influences fetal development. In addition our previous work has already shown that our rodent models mimic human physiological changes during pregnancy.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal welfare is paramount in our studies and refinements will be introduced wherever possible. All animals will be monitored throughout the study by staff trained to work with animals across the lifespan. Food intake, bodyweight, general condition, demeanour and behaviour will be monitored for a minimum of once a week. Any deviation from the normal will be recorded and when appropriate we will discuss with staff within the facility and take appropriate action however animals that develop severe effects will be humanely killed.

With this project licence we aim to introduce further refinements:

a. Collection of large amounts of high-quality data using state of the art technologies, thereby substantially reducing the number of studies/animals that are required to meet the objectives.

b. Using functional imaging to reduce the number of animals.

c. All animals undergoing anaesthesia will be monitored daily for up to 72 hours post-procedure and will be provided with post-operative analgesia. This monitoring will assess welfare aspects such as general appearance, appearance of wound (where appropriate), spontaneous behaviour, provoked behaviour and weight as well as signs of dehydration and diarrhoea and is based on the FELASA guidelines for determining severity. Furthermore an acute 10% loss of bodyweight or loss of bodyweight greater than 10% which has not recovered in seven days will lead to rodents being culled humanely. This monitoring system has been approved for use by our institutional AWERB and is currently in place.

d. Group sizes in ageing experiments will increased to accommodate for loss of animals due to old age, alongside longer drinking spouts being utilised. As the animals age we will increase the number of days the animals are monitored for food intake, bodyweight, general demeanour and behaviour to three days a week.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Throughout the project we will make sure to consult best practice guidelines such as the PREPARE guidelines, the Fund for Replacement of Animals in Medical Experiments (FRAME), NC3R website for experimental design and ARRIVE guidelines as well as LASA publications.

We will also keep up to date with the latest non-animal research models to ensure where possible, non-animals based experimental models are adopted (altweb, http://altweb.jhsph.edu).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As a group we subscribe to the NC3Rs monthly email alerts which keeps updated to the most recent developments leading to refinements of our methodologies. We also receive regular updates on welfare from the University of Nottingham Biomedical Research Unit, and we have bi-monthly meetings to discuss publications to discuss best practice and alternative methods which could refine, replace or reduce the number of animals we need. As PI I have attended NC3R workshops and have applied for grants.

165. The Role of Host Genetics, Intestinal Structure and Microbiome Diversity in Chicken Gut Health

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Broiler chickens, gut health, dysbiosis, selective breeding

Animal types	Life stages
Domestic	fowl (Gallus gallus domesticus) juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Modern broiler chickens reared for meat commonly suffer from poor gut health. We aim to define early life physiological and microbiological markers that associate with good intestinal health and can be used in broiler breeding programmes or diagnostics to improve gut health and reduce reliance on antimicrobial drugs.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
More than 72 billion broiler chickens are produced in the world every year, most to provide meat for human consumption. Selection for performance (e.g. body weight gain, food

conversion ratio) has been intense and in some examples unintended consequences have included predisposition to enteric dysbiosis. Enteric dysbiosis is a common condition where populations of bacteria and other microorganisms in the gut become unbalanced. Dysbiosis compromises broiler productivity and welfare, increasing the occurrence of disease, poor litter quality and lameness traits, as well as slaughterhouse condemnation resulting in poor welfare and wasted chicken production. Enteric dysbiosis is commonly prevented or controlled by routine application of antimicrobial prophylaxis, incurring a high requirement for antimicrobial use in chicken production. However, the use of antimicrobials in livestock production has been associated with selection for drug resistance in bacteria that might compromise human health. As chicken producers strive to reduce antimicrobial use alternatives are required, for example selective breeding to improve gut health. The well-established pyramid structure of broiler chicken production is highly amenable to selective breeding, with elite pedigree stock representing less than 0.0001% of chickens produced annually. Crossbreeding and amplification through great grand-/grand-/and parental generations produces ~4 million broiler chicks per pedigree female over a four-year cycle. Thus, improving gut health at the elite pedigree level will exert an enormous influence on farm-level broiler welfare and productivity, as well as reducing antimicrobial use. Here, we aim to identify a range of biological markers that can be used to identify individuals with good gut health from an early age to prioritise their use in selective breeding programmes.

What outputs do you think you will see at the end of this project?

The work will generate new data and knowledge about (1) fundamental biology of commercial broiler chickens, (2) the occurrence and consequences of poor chicken gut health, and (3) microbiological, physiological and transcriptomic markers that associate with good/bad gut health. These outputs are

also worthwhile in their own right because they contribute to understanding of the health of chickens and their microbiomes. The work will be published in peer-reviewed and industry journals, supporting the career development of the PhD students and early career postdoctoral scientists working on this project. The work is also essential for more targeted specific objectives:

Improved understanding of enteric dysbiosis in broiler chickens can be used to develop phenotypes associated with gut health that correlate with productivity and welfare. Such phenotypes can be used to improve commercial chicken lines by selective breeding and to highlight novel interventions.

Improved broiler chicken gut health is also expected to lower demand for antimicrobial intervention, reducing drug use in livestock production. Lower antimicrobial consumption will reduce selection for antimicrobial resistance in enteric and environmental microbial populations, and reduce antimicrobial flow into environments around chicken production systems.

Reducing the occurrence of ill health in chickens will lower the overall cost of poultry products, benefitting consumers as well as production and distribution networks.

The UK leads the world in breeding and production of broiler chickens through longstanding associations with companies such as Cobb-Vantress and Aviagen, both of whom have regional bases in the UK. Biomarkers associated with good gut health can be used to improve chicken lines and promote UK competitiveness.

Who or what will benefit from these outputs, and how?

The outputs are expected to provide benefits in the short, medium and long term. Short term
Staff and students working on the project will receive training in simulated farm and laboratory level settings, including a range of protocols that can only be applied with live animals and can also be used to answer a variety of experimental questions beyond the remit of this work.

Medium term

Chicken breeding companies will benefit from improved knowledge of enteric dysbiosis and identification of biomarkers that can be applied to improve the health and welfare of pedigree chicken lines. Such improvements will also improve their national and international competitiveness.

The national and international scientific community will benefit from improved understanding of enteric dysbiosis.

Long term

Improved chicken lines produced by selection for biomarkers of intestinal health will cascade down the breeding pyramid, increasing health and welfare of breeding and, ultimately, commercial stock.

Farmers/poultry producers will benefit from healthier stock, improving performance and profitability. Consumers will benefit from healthier, more cost-effective poultry products.

The general public will benefit from reduced antimicrobial use in poultry production, supporting reduced selection for resistance and lower risk of environmental contamination.

How will you look to maximise the outputs of this work?

All data produced from these studies will be published in Gold Open Access peer reviewed journals, as mandated and supported by the funding body (BBSRC). In addition to data, protocols and standards developed or applied will be described, providing resources and benchmarks for comparative studies. Data such as DNA or RNA sequences (e.g. microbiomes, host transcriptomes) will be submitted to open repositories, specifically the European Nucleotide Archive (ENA), linked to the DNA Data Bank of Japan (DDJB) and GenBank. Published studies will include results of null or unassociated measures. Results will be shared with peer audiences through national and international conferences (e.g. British and World Veterinary Poultry Association meetings).

Results and progress will also be reported in industry journals and magazines, as well as live events such as the Pig and Poultry Show, to ensure dissemination to relevant target audiences.

A series of collaborations will enhance outputs for the work. The work is supported by an industry partnering award (IPA), providing a direct link to industry. Both the applicant and partner also collaborate with researchers at national and international institutions that host world leading poultry biologists and geneticists.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): We expect to use up to 400 pedigree broiler breeder chickens in data generation, up to 150 SPF Lohmann Valo chickens and 350 commercial broiler chickens in validation. We may also use up to 100 interleukin (IL)-10 knockout chickens to assess gut integrity in an inflammatory immune environment. Please refer to the specific protocol for associated power calculations.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Chickens will be used throughout these studies, recognising that they are the target animal and not just a model. Enteric dysbiosis occurs naturally during production and will not be induced experimentally – we aim to identify markers that associate with good gut health. Clinical signs for enteric dysbiosis are difficult to define but include diarrhoea and lameness (bacterial chondronecrosis with osteomyelitis [BCO]), as well as non-specific signs such as lethargy, closed eyes and ruffled feathers.

A range of chicken types will be used, including (i) specific pathogen free chickens (Lohmann Valo) to permit accurate assessment of measures with minimal background variation, (ii) pedigree broiler breeder lines, representing the target population, (iii) commercial broiler chickens, providing real-life examples for validation, and (iv) a new interleukin (IL)-10 knockout (KO) chicken line. Chicks will typically be used up to six weeks of age (maximum of eight weeks old), recognising that dysbiosis is primarily a problem during the early rapid phase of broiler growth.

Typically, what will be done to an animal used in your project?

A range of chickens will be used, including specific pathogen free (SPF) Lohmann Valo and IL-10 KO chickens (Experiment type A) and pedigree or hybrid commercial broiler chickens (Experiment type B).

Experiment type A: controlled conditions parameter setting

SPF Lohmann Valo or IL-10 KO chickens will be used to define natural variation in candidate biomarkers for enteric dysbiosis in a ‘clean’ system with reduced background variation (e.g. controlled absence of specific pathogens, restricted host genetic diversity). These chickens are not expected to experience clinical dysbiosis, but will provide data on natural variation in enteric structure and function, as well as all candidate biomarkers.

- Typical experiments will include receipt of chickens at one day or ~two weeks of age followed by a seven-day settling-in period in groups of two to ten individuals in wire floored cages.

- Chickens may be placed in single bird cages for up to four hours to permit collection of faecal material from specific individuals, after which they will be returned to their group

cage. If a low volume of faecal material is required chickens may be cloacally swabbed as an alternative.

- Chickens may receive an oral dose of fluorescein isothiocyanate-dextran (FITC-d), followed by blood collection from a wing vein to assess FITC-d leakage from the gut. The process may be repeated on up to three occasions. Blood collected at the same time may also be used to assess a range of immune parameters including cytokine levels as well as bacteriaemia.

- Each study is expected to last a maximum of six weeks. Experiment type B: commercial conditions data collection
Pedigree or commercial broiler chickens will be used to identify candidate biomarkers in the target population expected to experience natural dysbiosis. Broiler chickens are not being used as a model.

- Typical experiments will include receipt of chicks at one day of age (industry standard) followed by a seven-day settling period in groups of two or more individuals.

- Most studies will accommodate broiler chicks in floor pens, although wire-floored cages may be used for the first two weeks of life if directly comparing with Lohmann or IL-10 cohorts.

- Chickens will be reared under commercial conditions (e.g. stocking density, feed, lighting regime) to assess the natural occurrence of enteric dysbiosis and associated phenotypes.

- Chickens may be placed in single bird cages for up to four hours to permit collection of faecal material from specific individuals, after which they will be returned to their group pen or cage. If a low

volume of faecal material is required chickens may be cloacally swabbed as an alternative.

- Chickens may receive an oral dose of FITC-d, followed by blood collection from a wing vein to assess FITC-d leakage from the gut. The process may be repeated on up to three occasions. Blood collected at the same time may also be used to assess a range of immune parameters including cytokine levels as well as bacteriaemia.

- Each study is expected to last a maximum of eight weeks. During this period it is anticipated that 10- 30% of pedigree individuals will experience enteric dysbiosis (3-15% for hybrid commercial broilers), a natural occurrence that is common to broiler chickens, although the level of occurrence may vary between chicken lines. Clinical signs for enteric dysbiosis are difficult to define but include diarrhoea and lameness (BCO), as well as non-specific signs such as lethargy, closed eyes and ruffled feathers. Chickens showing clinical signs of enteric dysbiosis will be removed from the study, culled, and sampled. Chickens showing clinical signs will not be retained.

What are the expected impacts and/or adverse effects for the animals during your project?

Enteric dysbiosis occurs naturally in up to 10-30% of commercial or pedigree broiler chickens when reared under commercial conditions (Experiment type B). Clinical signs are non-pathognomonic but include ruffling of feathers, paleness of comb and wattles,

permanently closed eyes, wet droppings, diarrhoea and/or bloody faeces, or reluctance to move. Detection of dysbiosis is the objective of the project and all individuals will be removed and culled for sampling when two or more of the signs are observed, or if a single sign persists for >24hrs. Euthanasia will not be delayed for any experimental or procedural reason. Chickens used in experiments of type A will not be expected to experience clinical dysbiosis, but will contribute to definitions of enteric structure and function.

Impacts and adverse effects of the procedures described here are expected to be mild. Blood collection from the wing vein can result in a localised haematoma but these are tolerated well. The risk is far lower than sampling from the jugular vein and consequences usually resolve within 24 hours (Livingstone, 2020). Oral inoculation is straightforward in chickens and very well tolerated.

Livingstone, M., CPD article: How to perform venipuncture in avian patients. Companion Animal, 2020. 25(10).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severities of the procedures carried out in this project are expected to be mild.

The natural occurrence of enteric dysbiosis is anticipated in up to 10-30% of individuals (current commercial expectations, data provided by the commercial partner), but will not be induced by any experimental procedure. SPF Lohmann and IL-10 KO chickens are not expected to experience enteric dysbiosis.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals are required for this project in the absence of a suitable in vitro/ex vivo alternative. Enteric dysbiosis occurs naturally during broiler production, primarily between three and five weeks of age. We aim to collect a range of data from chickens prior to the occurrence of dysbiosis that can be tested as predictors for good gut health.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives are not currently available for enteric dysbiosis. Compartmental models such as fermenters (gut lumen models), organoids and tissue explants represent incomplete systems and are not currently fit for purpose. The topic has been reviewed during preparation of this application (e.g. targeted searches of the published literature via PubMed, Web of Science and Google Scholar). Progress with explants and organoids has

been made in recent years (e.g. studies at the Roslin Research Institute; doi: 10.1038/s42003-021-01901-z), but lack the ability to replicate interactions between host, environment and microbiome.

Why were they not suitable?

Non-animal alternatives cannot be used to re-create the complex interaction between host, environment and microbiome required to assess enteric dysbiosis.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers requested have been estimated based on the expected occurrence of dysbiosis under production conditions and the numbers of individuals required in each phenotype group. Dysbiosis is anticipated in 10-30% of pedigree chickens (data provided by the commercial partner, supplier of the broiler chickens), and 3-15% of commercial broiler chickens. Our studies of enteric microbiome diversity indicate a requirement for at least ten individuals per phenotype to reliably identify microbiome type (termed ‘enterotype’).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We intend to use four different types of chicken during these studies to minimise biological variation and improve statistical power. First, using a known pedigree broiler breeder line provides reproducible appearance of enteric dysbiosis in 10-30% of individuals during normal production. The occurrence of dysbiosis is less predictable in most other chicken types. These pedigree birds are also defined by lower genetic diversity than hybrid commercial broiler chickens, improving reproducibility and permitting smaller group sizes (i.e. the top versus the bottom of the broiler breeding pyramid). SPF Lohmann Valo chickens provide additional control of microbiome variation with a similar genetically homogeneous background. Commercial broiler chickens will be used for validation, while IL-10 knockout chickens may be used to define the importance of the anti-inflammatory immune response.

We will use oral inoculation of fluorescein isothiocyanate-dextran (FITC-d) and subsequent measurement in serum to assess gut integrity and leakage, permitting repeated sampling from individual animals without a requirement for slaughter. The procedure is mild and reduces the number of chickens required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?
The studies proposed here have been based on current commercial knowledge and existing pilot data collected from broiler breeder populations in the field, where we have opportunistically sampled birds culled for management purposes. We have also

established considerable background data defining variation in measures such as microbiome diversity, transcription profiles and bacteriaemia from previous studies in the UK and overseas that can be used in power calculations (as outlined in protocol 1). Samples collected from these studies will be blinded for laboratory analyses. Tissues and data will be shared with other projects within the group and made available to others within the wider College community.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Industry data indicate that up to 10-30% of pedigree and commercial broiler chickens will experience enteric dysbiosis by five weeks of age when reared under standard commercial conditions in the

absence of antimicrobial prophylaxis. For this reason, we will not include any experimental procedure to induce dysbiosis – we will re-create standard farm conditions. The procedures listed here (oral inoculation, blood sampling, and cloacal swabbing) are well established and selected to minimise the need for invasive procedures. For example, final blood sampling will be undertaken immediately post- mortem rather than from live birds to minimise the number of procedures per individual.

Why can’t you use animals that are less sentient?

The study of enteric dysbiosis in chickens cannot be accurately replicated in any other less sentient animal.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Chickens will be habituated to experimental staff from arrival, including routine ‘pen walk throughs’ that will be used to detect chickens experiencing enteric dysbiosis.

Based on experience gained in recent studies with commercial broiler chickens it is clear that enteric dysbiosis is most likely to occur between three and five weeks of age. We will increase monitoring during this period.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow ARRIVE guidelines for experimental reporting in study design to ensure the most relevant and transparent experimental design. We will also apply community consensus guidelines for reporting (e.g. adapting the human microbiome STORMS

checklist to poultry; Mirzayi et al., 2021) to study design to ensure all sampling is fit for purpose.

We will follow expert guidance on husbandry from our commercial partner, ensuring that best practices are always followed.

Mirzayi C et al. (2021) Reporting guidelines for human microbiome research: the STORMS checklist. Nat Med 27(11):1885-1892.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The applicant and all team members who will work on the project regularly interact with the NC3Rs, checking the website regularly and attending seminars and webinars when they occur. The host organisation is also very active in dissemination of 3Rs relevant news, providing training and updates via a newsletter, emails and online notifications.

166. Fistulation of Ruminants to Remove Ruminal Content to be Used for In Vitro Evaluation of Anthelmintics and Antimicrobials.

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Anthelmintics, Fistulation, In vitro, Artificial, Reduction

Animal types	Life stages
Cattle	juvenile, adult
Sheep	juvenile, adult
Goats	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to create a fistula into the rumen of an animal to enable the removal of rumen contents for use in an artificial rumen, which will be used for the in vitro evaluation of potential new antimicrobials and anthelmintics, or safer, more effective versions of existing products.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The fresh rumen content is required to ‘seed’ an in vitro artificial rumen to aid and develop drug discovery and drug dynamics. It is preferable to test new products in vitro prior to animal testing. There is considerable research into replacements for antimicrobial use and also into replacements for anthelmintics where resistance is an increasing issue. This will hopefully lead to a reduction in animal usage for drug development trials going forward.

What outputs do you think you will see at the end of this project?

The fistulated animal(s) will be used to remove rumen contents to ‘seed’ an in vitro artificial rumen to aid and develop drug discovery and drug dynamics. This will hopefully lead to a reduction in animal usage for drug development trials going forward. This will lead to new and reformulated drugs to combat animal disease and increase animal welfare status on UK farms.

It will also create financial income for the company which will enable us to carry out further contract research projects involving parasites. We contribute to fundamental research which is used at world class institutes via the publication of scientific papers.

Who or what will benefit from these outputs, and how?

Animals, pharmaceutical companies, researchers, farmers and the wider economy will all benefit from the research carried out. The in vitro testing facilities provided may result in new and novel products

being brought to market to help alleviate many different animal diseases with reduced animal numbers required. Therefore, this benefits the animal’s wellbeing and health, farmers see improved yields and pharmaceutical companies see profits by marketing their product. This will also enable us to offer in vitro work as a service to others which may include research institutes and companies in the very early stages of drug discovery and development. Human health benefits from access to safe food and/or veterinary interventions. These factors combined stimulate and help the wider economy to grow within the UK. We earn income that assists the maintenance and development of our facilities.

How will you look to maximise the outputs of this work?

We will work with companies, institutes and charities to disseminate the study / experimental findings, publish research papers, attend relevant conferences and we also play an active role in creating guidelines in this area of research.

Species and numbers of animals expected to be used

- Cattle: 20
- Sheep: 20
- Goats: 20

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.
Fresh rumen contents (~2hours) is required to 'seed' the artificial rumen before trialling substances in it. Therefore only ruminants with a fully developed rumen can be used for this project. It is important to maintain the same rumen contents throughout an experiment for consistency and to reduce scientific variables.

Typically, what will be done to an animal used in your project?

The animal will have an area of skin shaven on its left dorsal abdomen or left flank over the rumen. A surgical fistula will then be created between the rumen and the skin by a competent Personal Licence holder who is also a veterinary surgeon with extensive experience in large animals. The sedation used will be moderate and most likely Rompun (xylazine) with a local anaesthetic given in an inverted L block on the animal. A dairy breed of calf will be selected to ensure minimum muscle covering over the rumen. Stay sutures will be put in place holding the skin to the rumen wall prior to piercing the rumen. Continuous or a series of stitches will then be placed around the circumference of the fistula to hold the skin and ruminal wall together. The aim will be to create a fistula that is approximately 3 fingers in width. The wound will be closely monitored and cleaned in the days following the procedure. Once the wound is suitably healed rumen contents will be removed from the animal with the aid of uterine forceps or similar. The fistula will remain in place until the animal is euthanased.

What are the expected impacts and/or adverse effects for the animals during your project?

- Pain at the fistula site following surgery – short term
- Discomfort

Possible complications following surgery:

- Off feed
- Weight loss
- Infection / peritonitis
- Wound dehiscence
- Fistula close-up
- Ruminal stasis

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We anticipate that the animals will experience short-term pain in line with the moderate severity category guidelines immediately after the fistula procedure.

Animal type	Number	Life Stage
Cattle	20	Adult and Juvenile (6 months+)
Sheep	20	Adult and Juvenile (6 months+)
Goats	20	Adult and Juvenile (6 months+)

What will happen to animals at the end of this project?

- Kept alive
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Fresh ruminal contents is required to ‘seed’ the artificial rumen. The only source of this is from ruminating animals. Rumen contents can be obtained from a commercial abattoir, however this has a number of implications. Firstly, the rumen contents would not be consistent throughout an in vitro experiment as very fresh rumen contents are required, secondly there are a number of legalities with removing animal by-products from a commercial site and finally there is the biosecurity aspect to consider associated with bringing rumen contents from cattle of unknown origin.

Which non-animal alternatives did you consider for use in this project?

This project is seeking to reduce animals used for drug discovery and drug dynamics.

Why were they not suitable?

N/A

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals required is based on 1 animal being required to ‘seed’ the artificial rumen per species.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We performed a harm, benefit analysis to weigh up the pros and cons of using rumen content from a commercial abattoir against using contents from a fistulated animal onsite. It was agreed an onsite animal is preferable from a scientific and biological stand point.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Running multiple studies in parallel to maximise the usage of 1 animal. Do preliminary work using rumen contents from a commercial abattoir before committing to a full scale experiment. Looking at previously published work within the same area.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animals used are the intended target for each veterinary substance that will be trialled in the artificial rumen and therefore their rumen contents and microbiota is essential in understanding how the substance reacts in the rumen. The species involved are: cattle, sheep and goats (ruminants).

Models are designed to minimise severity as far as possible to achieve meaningful results.

Why can’t you use animals that are less sentient?

The animals must be at a life stage where their rumen is fully developed and working properly.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The animals used are the required species for the veterinary substance being trialled. All models will have welfare at the centre of their design. In addition, animals will have a heightened level of observations immediately after and in the days following the creation of the fistula. Our establishment’s standard level of monitoring is a minimum of twice daily. Frequency of observations are increased on an as needed basis based on animal observations and behaviour. For example, observation frequency would increase to hourly or constant immediately following the creation of the fistula. Observations would normally be general health observations but these are supplemented with clinical observations by our Named Veterinary Surgeon (or similar), monitoring of temperature and other parameters as necessary. Pain relief will be administered following the surgery and in subsequent days.

If it is believed an animal is approaching and likely to breach the predetermined severity category then we have a well-established process. This includes seeking veterinary advice

from our Named Veterinary Surgeon or another veterinary surgeon, discussions with the Named Animal Care and Welfare Officers, Establishment Licence holder, Project Licence holder, Personal Licence holders and Study Investigator. This group of people decide the best next steps in terms of animal welfare. This could be either, immediate alleviating of suffering if irreversible clinical signs via euthanasia, treatment with, for example, analgesics and anti-inflammatories in order to relieve suffering, and increased monitoring if the animal is unlikely to breach the severity limit by the conclusion of the experimental time period if appropriate.

If an animal breaches the predetermined severity category for the planned work then immediate action is taken. This often includes rapid discussions with the Named Veterinary Surgeon, Establishment Licence holder, Project Licence holder and Personal Licence holders alongside the Study Investigator

and Named Animal Care and Welfare Officers. We would also, if possible, contact an Animals in Science Regulatory Unit inspector. The course of action could be immediate alleviation of suffering if there are irreversible clinical signs via euthanasia, treatment with, for example, analgesics and anti- inflammatories in order to relieve suffering, maintaining and closely monitoring the animal if the scientific need is justified and the end of the experiment is within a short time frame, if agreed by an Animals in Science Regulatory Unit inspector. All decisions are taken on an individual basis and will differ with treatments and species.

We will also continually review our procedures and refine as much as possible with guidance from, for example, Veterinary International Conference on Harmonization, European Medicines Agency , and other relevant organisations and published literature.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

N/A

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly reference the NC3Rs website, published literature, attend relevant conferences, and employees are members of The Royal Society of Biology and other relevant organisations.

167. Neural Stem Cells and Glioblastoma: New Understanding and New Therapeutics

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

stem cell, cancer, gene therapy, glioblastoma, therapeutics

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The most common brain tumour is glioblastoma and this is driven by cells which have similarities to the brain stem cells found in the nervous system. By comparing normal stem cells to those from glioblastoma we can identify new therapeutic targets, develop novel medicines, and test these new approaches for effectiveness.

There are two main aims of this project: 1) to define the key drivers and therapeutic vulnerabilities of brain cancer growth and; 2) to identify new small molecules or advanced therapies (e.g. cell or gene therapies) that could be developed further as new treatments.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
Brain cancer is now a leading cause of cancer-related deaths in the under 40's. It is responsible for more years of active life lost than any other human cancer. It is devastating for patients and their families. We have made very little progress in the last two decades in improving survival rates.

What outputs do you think you will see at the end of this project?

The program of work we propose will try to identify new therapeutic strategies to tackle the most common malignant brain cancer, glioblastoma.

There are two major benefits to this project: 1) those that increase fundamental knowledge of the biology of stem cells and cancer biology - specifically the identification of how brain cancer stem cells function and gene regulatory processes that might be new areas for drug discovery; 2) the specific application of new knowledge for the development of new therapies to treat patients with brain tumours
– particularly the most common and aggressive form, glioblastoma using either small molecule or gene therapy appraoches.

There is potentially broader value as our approaches and discoveries may reveal fundamental biological mechanisms that apply to all cancers, or could have relevance outside of oncology - for example in regenerative medicine and treatment of neurological disorders (e.g. multiple sclerosis, spinal cord injury, dementia).

Outputs will include publications on our research, collaboration with industry partners for development of new medicines, and engagment with the public to explain the challenges of brain cancer and the importance of research.

Who or what will benefit from these outputs, and how?

The short-term goals are to define new therapeutic targets – genetic studies that define essential genes that represent a vulnerability in the brain cancer stem cells. The medium-term goals are to prioritise those genes/pathways for which known drugs/inhibitors are available. The longer-term goal is to drive these drugs into preclinical studies and early clinical trials. The expected benefits therefore span across human cancers and have the possibility to result in new therapies. The fundamental discoveries in the basic biological mechanisms are harder to predict, but could have major impact in either the short or longer term by helping scientists understand the mechanisms of life.

How will you look to maximise the outputs of this work?

The outputs of of work will be prioritised for publication in leading scientific journals. We also work with government and charity funders to disseminate findings through media engagment, podcasts and patient days. These can include laboraotry tours. Many of our reagents and tools are disseminated widely to the reserach community as open access data sets or resources.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To study brain cancers we need to work with living animals to explore certain features of the disease. Most notably the immune system/blood interactions, infiltrative/migratory behaviour, interactions with blood vessels and impact on normal brain tissues of any novel medicine. These studies complement our work with non-animal based models in the laboratory culture dish. Glioblastoma is primarily an adult disease that emerges in the adult brain. We therefore work mainly with adult mice.

The mouse is the favoured animal species, as we can reliably transplant cells directly into the brain in regions that do not cause significant symptoms until the tumour has grown significantly. This means that we can track tumours in live mice and get a lot of information before the animals develop significant clinical signs relating to the tumour.

Typically, what will be done to an animal used in your project?

We will test new candidate drug-like molecules and therapies to see how they affect tumour growth and use the live imaging of tumour growth to monitor responses. Our studies in mice enable us to quickly determine which genes and pathways are involved in this infiltration, tumour growth, and relapse after anti-proliferative therapies. We will also model the disease by genetically engineering into normal brain stem cells a set of the most common glioblastoma-associated mutations, typically around 5-10 different pathways. These cells will then be injected into the brain to create the tumours.

The main procedure is to transplant human cells into the adult brain of mice that have been genetically modified to reduce their immune system; in this way the human tumours can form without rejection.
For immune system studies we use mouse brain tumour cells and inject into mice that are genetically matched so the immune cell-tumour intearctions can be explored.

For our studies of the small molecule (drug-like) therapeutics we deliver to the mice (typically either orally, or through injection into the bloodstream). For advanced therapies, such as viral based gene therapies, we inject the therapeutic agent directly into the tumour mass.

All mice are kept in individually vented cages (IVCs) to prevent any risk of infection. We have all of the state-of-the-art equipment needed for this project and close contact to trained veterinary staff who provide input and advice on all procedures. By tracking tumour growth in live animals we can collect a lot of data prior to any symptoms emerging and monitor accurately when tumour burden becomes too high. For our 5 year project we anticipate using around 1000 mice per year across the 5-10 different project areas (a team of 10-20 people).

What are the expected impacts and/or adverse effects for the animals during your project?

We anticipate that the mice will develop brain tumours. For some experiments, we can monitor tumour growth before symptoms develop. In cases where this is not possible the mice are closely monitored and humanely sacrificed when clinical signs beyond mild are detected. The transplant procedure itself may causes some discomfort; however, the brain has no pain receptors and mice rapidly recover from the procedure without any deficits. This is a moderate level of severity. Also, mice are given analgesia and the procedure is performed under general anaesthetic. Once tumours have developed animals will be killed humanely and their brain and tumour tissue will be analysed by detailed histological and molecular tests.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severities are considered to be moderate (~90%) and mild (~10%) for all procedures. The novel therapeutics may have unexpected consequences or severities, but in this instances we will use careful dose escalation studies, with more frequent monitoring, similarly to those used in human clinical studies.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to explore tumour cell behaviour in the appropriate tissue microenvironment (e.g. blood vessels, nerve tracks and cerebral spinal fluid contact) as these are known signals that affect tumour cell proliferation, dormancy and specialisation. It is also vital to study tumour cell behaviour in a whole brain, as we can monitor the extent of infiltration and spread. The physiological effects of anticancer drugs require a whole animal study, as small moleucles can be metabolized by the liver altering their activity and pharmacokinetic and dynamic properties. Finally, we need to study how well drugs can enter the brain and cross the blood –brain barrier. This is not possible in laboratory culture.

Which non-animal alternatives did you consider for use in this project?

Cell culture models are being used by our laboratory and are valuable for exploring the tumour cell intrinsic properties. However, to understand tumour growth and interactions with normal brain tissue these are not suitable in most instances. We are considering co-culture assays to explore immune cells interactions, but these often have limited value as a whole organism is needed for processes such as immunological memory and recruitment of cells from the bone marrow to be studied.

Why were they not suitable?

Studies in the cell culture dish often do not enable the long term monitoring of cell behaviours and fail to recreate the cellular complexity that exists in the live adult mouse brain.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have carefully selected the group sizes and numbers required based on anticipated statistical power required. All experiments performed in the mice will have had significant cell culture experiments performed to generate the initial hypothesis. We will use non-invasive imaging techniques in some instances to enable tracking of tumour growth and reduction in numbers of mice, as more accurate quantitative data is obtained. We are where possible exploiting organotypic brain slice cultures to assess tumour aggressiveness which can reduce the numbers of animals further. We anticipate 1000 mice per year to be used. This is approximately 100 per working month. In a typical month we may perform 4 or 5 transplants procedures or testing of therapeutic agents.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In the experimental design stage of the project, we have made plans to perform pilot studies where possible before planning for large cohorts. All experiments will be conducted according to the PREPARE guidelines https://norecopa.no/prepare . For most experiments we have more than 10 years of experience in performing the procedures and have constantly reduced and refined numbers of animals based on known survival rates and statistical analysis of past studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will implement efficient breeding procedures and will additionally perform well-designed pilot studies whenever possible to assess the potential for publishable results. We will complement wherenever possible these animal studies with the cell culture and co-culture models, which is the major experimental approach used by our laboratory. The possiblities to use these depends on outcomes from the animal studies, and is therefore highly iterative based on results that emerge (which cannot be predicted in advance).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are suitable for studies of brain tumour experiments as there is considerable knowledge of mouse brain physiology, histology and molecular biology. Mouse is the preeminent model for genetically modifying cells and we can therefore make use of many available transgenic strains. We have taken many steps to refine our procedures over the past few years. For example, using inhalable gas anaesthetics and smaller needles reduces tissue damage and recovery times. We also use analgesia during surgical procedures. We will use humane endpoint for testing new drugs, in line with guidance from local vets, with close monitoring and killing of any animals with signs of large tumours.

Why can’t you use animals that are less sentient?

As the goal of our research is understanding the complexities of brain cancers and development of new therapies, it is vital that as many features of the human disease as possible are accurately

modelled. e.g. the blood brain barrier. Also the time course of tumour growth is over weeks or months - and this is not possible to model accurately using lower vertebrates or invertebrates.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Any of the studies involving surgical or invasive intervention will adopt appropriate pain management and post-operative care.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will stay up to date on best practice guidelines set forth and regularly updated from the NC3Rs website (Guidance on the Operations of ASPA - https://www.nc3rs.org.uk/) and the guidelines for the welfare and use of animals in cancer research in particular. https://www.nature.com/articles/6605642

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about the advances in the 3Rs by attending informational events provided locally and provided by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

168. Discovering and Testing New Treatments for Kidney and Bladder Disorders in Children and Adults

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

bladder disorders, congenital abnormalities of the urinary tract, kidney disease, regenerative medicine, therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to identify new treatment strategies for diseases of the urinary tract in children and adults. These therapies will then be tested in pre-clinical models of kidney and bladder disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
The work in this project licence aims to develop new treatments to improve the quality of life of patients with kidney disease. This is important as one in ten people around the world

have chronic kidney disease. Critically, a subset of these individuals have end-stage kidney disease, a state in which kidney function is so poor that life cannot be sustained without renal replacement therapy. Worldwide, around two and a half million such patients are currently alive either being treated with long-term dialysis or being in receipt of a functioning kidney transplant. Despite these interventions, which are costly to health services and place a high burden on patients, it is estimated that two million individuals with kidney disease still die each year, unable to access dialysis or transplantation. This means new treatments for disorders of the urinary tract are urgently needed.

What outputs do you think you will see at the end of this project?

The new scientific knowledge from this project will be:

(i) the identification of new therapeutic targets for treating congenital and acquired urinary tract disorders.

(ii) generating new knowledge to understand the biological processes which underlie disorders of the urinary tract, particularly focusing on the role of blood and lymphatic vessels and immune cells.

(iii) to establish methods which target new treatments to the urinary tract and determine their effectiveness in relevant pre-clinical models.

(iv) to determine if regenerative medicine strategies can improve models of progressive renal failure.

This new knowledge will be demonstrated in the form of peer-reviewed publications. We will also disseminate our findings through presentations at conferences both locally, nationally, and internationally.

Some of the outputs of this licence may also lead to new intellectual property (IP) such as the identification of new therapeutic targets for kidney and bladder disease and novel technologies to deliver therapies to the urinary tract.

Who or what will benefit from these outputs, and how?

Several groups will benefit from the research outlined including:

(i) Children and adults with diseases of the urinary tract. There is an urgent need to design new therapies to improve the life of patients with kidney or bladder disease. Our work is designed to meet this need and identify and test new treatments for kidney and bladder disease. If successful, this may lead to clinical trials of new treatments in the next five to ten years. This would have a significant impact on patients with kidney or bladder disease.

(ii) Kidney and bladder researchers. Our work will identify new biological pathways and molecules that are involved in the progression of kidney and bladder disease. Additionally, we will explore the contribution of the vasculature and associated immune cells to the healthy and diseased urinary tract. Finally, we will establish if new treatments using therapeutic molecules or regenerative medicine can be used as treatments for urinary tract disorders. This information will be of high interest to researchers within the kidney and bladder fields.

(iii) Vascular biologists and immunologists. A portion of the work in this licence will examine the role of the vasculature (in particular lymphatics) and associated immune cells in the healthy and diseased urinary tract. This information will be of interest to other vascular biologists and immunologists working in other organ systems who could apply the information discovered in the urinary tract to other contexts.

(iv) Developmental and stem cell biologists. Part of this project will test whether regenerative medicine approaches could improve models of progressive renal failure. This translational approach will be of interest to other stem cell biologists and may provide important information and technologies which could be used to treat different diseases using cell-based therapies.

(v) Pharmaceutical industry. One of our main approaches will use non-biased technologies such as single-cell RNA-sequencing (scRNA-seq) to evaluate gene expression in healthy and diseased urinary tracts. This data will allow us to identify new molecules and biological pathways which can be targeted with treatments to improve urinary tract disorders. This information will be of interest to the pharmaceutical industry. They will also be interested in our novel approaches to target therapies to the kidney and bladder and our strategies to use cell-based therapies as treatments for urinary tract disorders.

(vi) Current collaborators with our group. We have collaborative links with academic and industrial researchers in the UK and internationally with expertise in renal biology, vascular biology, development, regenerative medicine, and gene therapy. The work in this licence will identify best practices in the techniques underlying our animal work which could be applied to other contexts, generate new future research directions with our current collaborators and may kick-start new collaborative interactions.

How will you look to maximise the outputs of this work?

To maximise the outputs of our work, we will ensure that all our publications will be uploaded as soon as they are accepted on our local university server which can be accessed by other users immediately. Alongside including detailed methodology of all experiments and raw numerical data within the supplementary material of papers, we will deposit image analysis and bioinformatics pipelines in open access forums such as GitHub so they can be replicated by other investigators. The results from scRNA-seq experiments will be deposited on NCBI's Gene Expression Omnibus (GEO) repository.
Data and protocols (including unsuccessful approaches) will be shared with our extensive network of collaborators increasing the value of the experiments being performed in this licence.

Our laboratory also has an established system for disseminating our research findings to non- academic beneficiaries and the public by:

(i) regularly updating our laboratory website and Twitter handle

(ii) using central systems at our university (public engagement unit and translational research office) to network between researchers, pharma, and the biotechnology industry

(iii) engaging with the media, funders, and patients/public.

To maximise any outputs which might lead to new IP we will consult the commercialisation company of our university, who have a demonstrable track record of success, including raising funds for spin-out companies and out-licensing and partnering IP to commercial success and societal benefit.

Species and numbers of animals expected to be used

- Mice: 27000
- Rats: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The majority of our work will be performed in mice. Mice are the lowest species in which suitable models can replicate most features of human urinary tract disease; in addition, gene manipulation, including temporal- and spatial- specific deletion and overexpression in the urinary tract is currently feasible and will be informative in our studies of renal disease. A few selected experiments will use rats, particularly looking at complementary strains (such as for polycystic kidney disease), or if surgical interventions are required where their larger size makes them more suitable. As our work aims to

identify and test new treatments for both children and adults with kidney and bladder disease we will use animals throughout the life-course from embryos, neonates, adult, and pregnant adults.

Typically, what will be done to an animal used in your project?

Most animals in this licence will be used for breeding to produce, maintain, and provide genetically altered animals. Offspring from these animals will be phenotyped in detail. This may involve: (i) the collection of blood; (ii) obtaining overnight urine in metabolic cages; (iii) injecting animals with tracing agents to assess blood supply and kidney structure and function and (iv) using non-invasive imaging (MRI, nuclear and photoacoustic imaging) to assess longitudinal changes in kidney function. Some animals will be administered chemicals or have surgery performed on them to induce kidney or bladder disease in the subsequent weeks and months after the protocol has been performed. Treatments will be provided to the animals to improve disease progression, some of which will involve administering biological molecules directly to the kidney or bladder by ultrasound technology or cell-based therapies using biocompatible scaffolds as a delivery platform.

What are the expected impacts and/or adverse effects for the animals during your project?

Potential impacts and adverse effects for the animals in this project licence may include:

(i) changes in appearance – lack of grooming, piloerection, hunched appearance, low tail carriage, pale appearance, and presence of milk in the stomach of neonatal mice.

(ii) alterations in behaviour – less mobile, reduced interaction with peers, weak responses to provoked behaviour.
(iii) loss of body weight

(iv) pain following injections or surgery.

All animals will be carefully monitored, and a score sheet completed for each neonatal and adult animal to assess their health. Animals will have appropriate anaesthetics and pain relief to minimise discomfort, but any becoming overly distressed will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most animals in this licence (around 70%) will experience a sub-threshold level of severity with their purpose being breeding to produce, maintain and provide genetically altered mice. 5-10% of the animals on the licence will experience a mild phenotype. This may be due to them undergoing procedures such as urine collection in metabolic cages, blood sampling and injections. These animals may experience transient pain at the time of the procedures. The remaining 20-25% of mice will experience a moderate severity. This may be due to the procedure being undertaken such as a surgical operation or signs of disease such as a decline in kidney function due to the animals having specific genetic alterations and/or induced renal disease.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The overarching aim of this project is to identify and test new treatment strategies for diseases of the urinary tract in children and adults. Identifying and delivering new treatments in the context of kidney and bladder disease is a complex process which involves not only interactions between different cell types but is also dependent on circulating factors and blood supply. These parameters cannot yet be fully replicated in cell culture systems meaning whole animal experiments are still required.

Which non-animal alternatives did you consider for use in this project?

Our laboratory has established a complementary programme of non-animal alternatives which we consider before embarking on any study involving animals. Our approaches range from two- dimensional culture of human and rodent kidney and bladder cells, explant culture of developing rodent kidneys and bladders, 3-dimensional models of kidney disease using gene editing and culture of mini organs (kidney and blood vasculature) in a dish from human stem cells.

Why were they not suitable?
Although significant progress has been made in developing new models to replicate the urinary tract in culture, experiments in whole animals are still required to identify and test new therapies for urinary tract disease. The progression of kidney and bladder disease involves interactions between different cell types, circulating factors and blood supply. This is still yet to be replicated in the most advanced 3- dimensional urinary tract organoids currently available which still lack both a ureter which is necessary to drain urine and the correct patterning of the renal vasculature which is critical for the functions of filtration and reabsorption in the kidney. They are considerable smaller with the most advanced organoids containing 100 nephrons (the functional unit of the kidney), several magnitudes smaller than the human kidneys which contains 1-2 million nephrons. Currently, although flow can be added to organoids this does not replicate the blood supply and all the circulating factors the urinary tract in health and disease is exposed to. It is also not possible to fully replicate kidney and bladder diseases in culture. These either develop over a long time (for example diabetic kidney disease), involve stimuli which induce an immune response in the host (glomerulonephritis) or need to be induced by surgical interventions such as bladder obstructions. These conditions can still only be fully replicated in whole animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of overall animals estimated in the licence is based on our previous experience over the last ten years. During this time, our group of 15 researchers have performed a diverse array of animal research experiments that broadly corresponds to the numbers of animals we are proposing for this PPL to cover in the next 5 years. We anticipate that the size of group, level of funding and amount of experimental animal work will remain the same over the next five years of this licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

(i) comprehensive literature and database review. This will prevent unnecessary production of genetically altered mice or duplication of previous work. Sources of information will include Pubmed https://pubmed.ncbi.nlm.nih.gov/, UK mouse locator network https://mouse-locator.crick.ac.uk/ and the international mouse strain resource http://www.findmice.org/.

(ii) consider in vitro alternative approaches. Our laboratory has a complementary programme of non- animal alternatives to investigate the kidney and bladder in health and disease. Before embarking on any animal study, we will explore whether our research questions can be answered in cultured cells, explant culture or organoids.

(iii) design experiments to generate biologically important results using the minimum number of animals. We will utilise the NC3Rs experimental design assistant to help generate a protocol for each study. This will include:

(a) a statement of the objective(s)

(b) a description of the experiment, covering such matters as the experimental treatment and number of animals in each group. Power calculations will be performed to determine sample size.

(c) an outline of the data obtained and how it will be analysed. This may include a sketch of the analysis of variance, an indication of the tabular form in which the results will be shown, and some account of the tests of significance to be made and the treatment differences that are to be estimated.

If required, our group also has access to local statistical support services who can provide further advice on study design, sample size calculations and statistical analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Additional steps to optimise the number of animals used in the project will include:

(i) refining breeding programmes wherever possible, to reduce numbers and produce only the genotype required e.g., using homozygous breeding pairs, intermittent breeding strategies to maintain colonies, archiving strains not being used.

(ii) performing pilot studies to optimise doses in animal models or therapeutic interventions (which may vary in different background strains and genetically altered mice) in a small number of animals before moving on to more substantial experiments.

(iii) where possible performing clinically translatable longitudinal assessments using high-resolution non-invasive imaging allowing the same animal to be examined over time. This will occur in experiments where the effect of therapeutic treatments on renal function and structure is considered and will minimise the number of animals used in the project.

(iv) using powerful computer analysis technologies to provide quantitative information to answer our research question with the minimal number of mice.

(v) sharing tissue. Our group is based within a department where there are investigators interested in brain, eye, ear, gut, heart, and lung development. We will utilise this network to share any tissues that may be of interest to others studying other organ networks. In addition, we have long-term collaborators with experts in the lymphatic field who may be interested in examining material from our genetically altered mice maximising the resources from the animals in this licence.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.
The animal models and methods used in this licence have been chosen to achieve our scientific aim of identifying new targets for urinary tract disorders and subsequently evaluating these therapies whilst minimising suffering.

To achieve this, we need pre-clinical models which faithfully replicate the pathophysiology of disorders of the urinary tract. Mice are the lowest species in which suitable models can be assessed to replicate most features of human urinary tract disease; in addition, gene manipulation, including temporal- and spatial- specific deletion and overexpression in the urinary tract is currently feasible and will be informative in studies of renal disease. A few selected experiments will use rats, particularly looking at complementary strains (such as for cystic kidneys), if surgical interventions are required where their larger size makes them more suitable or when preliminary data has been found predominately in this species (in the case of dietary manipulation).

We are very experienced in all these models and understand the time-course of disease and any adverse effects. Animals are carefully monitored by bespoke scoring system established in our laboratory for each experiment to prevent unnecessary harm. Techniques are refined and experiment- specific protocols outlined following discussions with the NACWO and NVS. Prior to beginning any experimental study, pre-study forms are submitted and kept on file with the NACWO to ensure best experimental practices. Experiments are completed in as short duration as possible whilst achieving our scientific goals to reduce unnecessary suffering, pain, or lasting harm to the animals.

Why can’t you use animals that are less sentient?

Pre-clinical models that faithfully replicate the pathobiology of childhood and adult urinary tract disease are not available in less sentient animals. Many of these occur in adults so using animals at a more immature life stage is not suitable. Animals that are terminally anaesthetised are not suitable for our work as this does not provide an opportunity to observe disease progression or test therapeutic agents.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Prior to any procedure taking place, we will fully engage with our NACWO and NVS staff to refine our experimental design to minimise harm for the animals. To facilitate this process, a pre-study request form is completed outlining responsible staff, procedure steps, monitoring, humane end points and adverse effects. This will allow us to refine:

(i) staff training to make sure all users are fully competent in the required experimental techniques to minimise harm.

(ii) the provision of anaesthesia and analgesia to minimise harm of the procedures to animals

(iii) sites of injection, volume, and number to minimise harm to animals

(iv) duration of experiments to ensure they are completed in the shortest possible time to minimise any periods of pain and distress to the animals.

(v) monitoring procedures so animals are inspected by investigators and animal facility staff at least daily to ensure that there are no signs of ill health.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our starting point for ensuring that experiments are conducted in the most refined way will be to consult the 3Rs resource library https://www.nc3rs.org.uk/3rs-resources which contains best practice on handling, husbandry, sampling, anaesthesia, analgesia, and welfare assessments. If additional resources are required for specific procedures, then we will perform extensive literature reviews on the potential technical and pharmacological constraints of a new approach before developing the study design in the form of pilots, prior to larger scale studies being undertaken. At every step of our research design, we will fully engage our NACWO and NVS to help with refinement and following

experiments, reflect on the outcomes of any procedure to make technical refinements to ensure animal welfare.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our research team is regularly informed of advances in the 3R's by the NC3Rs regional programme manager. These are communicated through various channels including: (i) presentations at the local committee of animal users; (ii) updates on the Biological Services website of our university; (iii) monthly newsletters sent to animal users and (iv) annual events for animal users at our university. A further source for information is the 3Rs resource library https://www.nc3rs.org.uk/3rs-resources and regular NC3Rs meeting organised online or in person on experimental design and techniques, which members of the research team have and will continue to attend. Information on these 3Rs advances and events will be passed on to the research team by the lead investigator and any recommended refinements to improve experimental procedures implemented following discussions with the NACWO and NVS.

169. Assessment of Immunological Tools to Tackle Infectious Diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Vaccine, Infectious Pathogens, Antibodies, Cell Immunity

Animal types	Life stages
Mice	adult, juvenile, embryo, neonate, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to dissect the levels and mechanisms of immunity, elicited by novel or current vaccines; as well as the assessment and development of immunotherapeutic interventions to prevent diseases caused by current or emerging infectious pathogens, either communicable (i.e. SARS-CoV-2, HPV) or transmitted by an arthropod vector, such as mosquitos (i.e. Usutu virus, Zika virus, Chikungunya virus) or ticks (i.e. Tickborne fever virus, Powassan virus).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Diseases caused by current and emerging pathogens kill millions every year. Antibiotics, antivirals, and preventive epidemiological interventions help at decreasing deaths. However, hospitalisation and treatment of diseases inflict economic damage at the global scale. Currently, vaccination programs can save more than 3 million people per year. The societal importance of vaccine development and the exploration of immunological mechanisms of protection against prevalent and emerging pathogens is evident with the sudden appearance of Zika virus in 2015 and with the recent emergence of coronavirus SARS-CoV-2. The relevance of vaccine research and their clinical implementation is of pivotal importance to decrease deaths and economic burden on health systems. Equally important, since most of the recent outbreaks or pandemics came from wild animals, this work will also inform and help towards the suitability of such vaccines for the veterinary field.

What outputs do you think you will see at the end of this project?

a) Highly potent and new vaccines.

b) Improved vaccination regimes.

c) Elucidation of novel mechanism of protection against pathogens.

d) Identification of novel targets to improve immunity.

e) Identification of novel targets for serology or clinical detection of diseases.

f) Generation of intellectual property related to a,b,d and e.

Who or what will benefit from these outputs, and how?

While this work will inform our own vaccine developments and researchers across our Institute, the publication and presentation of data generated from this project will also inform other researchers and

help to advance the overall knowledge in the field of vaccine development. The results and data generated will be shared in scientific meetings, conferences, scientific board meetings to broad the knowledge of the mechanisms of immune system to control pathogens and to inform immune correlates of protection generated by vaccination.

Outputs of this research will be fully available to the whole scientific community and wider public, we will ensure the publication of this work will be ‘Open Access’.

In the medium term, the discovery of novel vaccines or novel vaccination regimes will inform the implementation of Phase I clinical trials and will provide the seed for starting manufacturing material.

In the long term, vaccines developed under this project would be translational and hopefully deployed in the clinical setting to prevent infections.

How will you look to maximise the outputs of this work?

a) The project will allow the improvement of vaccines or new development of vaccines. With this, some vaccine developments may not render effective immunogenicity or

efficacy. Such negative results will be shared to collaborators to further inform a better understanding of the requirements of an efficacious vaccine.

b) Low or medium efficacious vaccine data will be published in peer-review journal papers which are dedicated to disseminating such outputs.

c) Our establishment actively participate in animal welfare workshops and conferences (i.e. The 3R research day), in which we share and discuss technical experience for further improve the research based on animal work.

Species and numbers of animals expected to be used

- Mice: 18000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice have been used for many years to characterise immunological responses, as well as for the design and induction of human-like disease symptoms. Importantly, just like humans, mice have cellular receptors, which serve as molecular docks for pathogens, such parasites, viruses or bacteria. The studies proposed in this project will take advantage of the mouse immune system as it is similar to that of a human. Hence, mice are the most ideal preclinical model to test vaccine efficacy and immune correlates of protection against infectious diseases. In exceptional circumstances, we may need to ensure that our vaccines are able to induce long-term immune responses. Hence, some mice groups vaccinated from 4 to 8 weeks of age may be followed up to study their immune system kinetics across and up to 24 months of age; aged animals will not be vaccinated.

Typically, what will be done to an animal used in your project?

The following proposed procedures are:

1) Immunisation through injection of vaccines, on single injection or followed by 1 or 2 boosters.

2) Blood sampling pre immune, and post immune bleeds are expected and will not surpass the blood volume limits at any given time or during the duration of the experiment.

3) Mice will be exposed to anaesthetics during immunisation procedures.

4) After termination of experiments, mice will be humanely killed, followed by organ harvesting.

5) For efficacy experiments, vaccinated animals will be exposed to pathogens via one injection of viral particles (such as Zika or another arthropod-borne or communicable virus). Such challenge experiments have a well-defined humane endpoint.

What are the expected impacts and/or adverse effects for the animals during your project?

For immunisations, injection of vaccines may cause a mild inflammation on the injection site and such inflammation will resolve one day after the injection, or earlier; this mirrors the mild symptoms expected after 48 hours after vaccine administration in humans.

For pathogen challenge experiments. Adverse effects increase substantially after the humane endpoint: When testing the efficacy of arthropod-transmitted vaccines, we monitor mice for virus present in the blood, with animals displaying mild systemic signs of disease (piloerection, hunch, ruffled fur) at the later days after virus challenge. However, for zika virus and other arthropod borne viruses; in some cases, and depending of the mouse strain and virus strain, animals will display weight loss. Such weight loss will be monitored very closely after viral challenge and with multiple readings until reaching the humane endpoint. In general, bodyweight loss greater than 15% (typically by day 9 after challenge) will define a humane end point.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

20% for moderate and 80% for mild.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice display characterised immunological responses to vaccines and foreign antigens, as well as presenting human-like disease symptoms after pathogen infection. Hence, mice are the most ideal preclinical model to test vaccine efficacy and immune correlates of protection against infectious diseases.

Which non-animal alternatives did you consider for use in this project?

Our group and collaborators strive to design in vitro protocols or assays that can help to determine whether the sera, or cells from animals, previously exposed to a pathogen or previously vaccinated against a specific pathogen, are able to inhibit or neutralise such infectious agent. Such in vitro assays (virus neutralisation) are indeed useful to gain an understanding of how effective a vaccine can be.
However, this unfortunately does not offer a high impact in reducing the number of mice to use in this program of work.

Why were they not suitable?

The above in vitro assays function only as surrogate of protection and will help to explore or determine the potency of vaccinated mice. However, mice are still needed to replicate the full immunological process after vaccination. For vaccine development, we need a fully developed immune system that can respond to a foreign antigen. Such systemic response in a mammal includes the activation of immune cells, their regulation, the orchestrated gene expression machinery to produce cytokines or antibodies, as well as the mechanism of immune memory in the event of a subsequent exposure. This complex immune system exists in mice and this resembles that of a human. As a consequence, there is no possibility to use non-animal alternatives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers are estimated based on the number of animals used in our previous PPLs.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our previous experience in our group and outcomes from our previous and current animal licences have allowed to design experiments based on:

a) The use of pilot studies which involved small number of mice and will allow a much-informed design of experiments in this project.

b) The use of the correct statistical power that will allow to find difference between groups.

c) A thorough selection of vaccine candidates, instead of testing several iterations of antigens or vaccines. We have implemented an in-silico and bioinformatics approach that allows the identification of better secretion properties of an antigen by modulating the hydrophobic anchors. Furthermore, vaccines will use only adjuvants that are validated for human use, hence reducing this factor will ensure the minimum number of groups per experiments.

d) The multidisciplinary skills of our groups and collaborators allows the identification of targets that can be used for vaccine development. This will save mice numbers as we would not need to test many antigens at once but only the more important targets in pathogen-host interactions.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

a) We will obtain sera from naïve mice by intracardiac bleeding and store serum at -20C, in small aliquots. Hence, for future experiments we will not need to use more naïve animal and this will help to optimise the total number of animals during the licence span.

b) We will aim to study the kinetics of the immune response before, during and after immunisations in the same group on animals. Sera will be stored at different timepoints: i.e. Pre-vaccination, 2 weeks after prime vaccinations and 2 week after second vaccination, for the same animal within groups. This will save number of mice to be used.

c) We will optimise breeding to produce only the genotype required and breeding colonies monitored closely to avoid inefficient and non-productive crosses. Samples for genotyping will be taken in timely manner so that the incorrect genotypes are not kept within the colony.

d) Sharing tissue: we will collect multiple organs from single experiments and share samples between groups/collaborators when possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use mice, as they are standard species for immunogenicity testing of vaccines. Mouse is a well characterised species in terms of their immunes system. In fact, the most relevant findings and mechanisms of immunity in humans have been discovered in mice. When testing the vaccine efficacy, we use strains of mice and viral strains in which the disease course is well defined, therefore the scientific readout can be reached at earlier timepoints in the disease course.

For immunisation purposes, injections using standard and well-defined routes of administration will be used and such routes of administration are currently used in the clinical setting. This will minimise pain and stress to animals. For challenge experiments, a clear and defined human end points will allow us to minimise discomfort and pain in great extent. Hence, a well-defined vaccine dose, a route of vaccination and well-defined model of vaccine efficacy ensures that mice will be free of long-lasting harm.

For bleeding purposes, we will ensure that mice are properly warmed up in heating chambers which allows the dilation of the tail vein. The operator will be sufficiently trained to achieve the right nick depth in the tail, so the bleeding is consistent during all experiments.

For challenge experiments, In the case of zika virus challenge model, the endpoint proposed in this licence are bodyweight loss greater than 15% This strategy will help to quickly determine the humane endpoint. However, through a collaboration with another establishment, I have analysed the temperature of mice after zika challenge (using a thermal sensor chip implanted in mice); and there is clear evidence that temperature fluctuations after the zika challenge could be used to determine a novel way to assess humane endpoints. Such effort will refine the challenge model and hopefully we can use it

to determine much earlier whether the animals need to be killed, before some other symptoms can occur.

Why can't you use animals that are less sentient?

Our main objective for this project is to explore mechanisms of immunity and the development of new vaccines against pathogens that affect humans, with a possible application to veterinary medicine.
Since mice possess similar immune system to that of a human being, we then can assess our vaccines or immunological tools. Unfortunately, we are not able to carry out our research in any other animal different of that a mammal. Of advantage, mice strains also offer the reproducibility of experiments and results and we are able to follow their immune response over time.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

1. We will constantly look for more refined alternative approaches. As the primary aim of our project is to develop vaccine for clinical translation, we will use adjuvants (or bioequivalent) that have been approved for use in humans.

2. We use inhalation anaesthesia, which minimise the need of mice handling and extra injections.

3. For the immunisation protocol and efficacy protocol, mice will be fairly monitored and in case they experience transient malaise, heat and moist food at a low-reaching level will be provided.

4. Our extensive vaccine development experience in our establishment enables the selection of most informative timepoints, hence we will decrease welfare costs.

5. We use short term anaesthesia when the route of injection can be painful, eg intramuscular injection.

6. Our typical route of vaccine administration is in-line with standard administration of vaccines in humans (eg intramuscular, intradermal, sub-cutaneous) and only use alternative routes of vaccine administration when targeting the response to specific sites.

7. For sensing body temperature, the implanted chip temperature sensor would not require surgical procedure.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

- Alternatives to Animal Experimentation Journal.

- The Handbook of Laboratory Animal Management and Welfare, Sarah Wolfensohn and Maggie Lloyd, Blackwell publishing, Third Edition (2003).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

- Through NC3Rs and our establishment 3Rs newsletters, and 3Rs meetings.

- By constantly monitoring the research related to improved models that could be implemented in our research.

- By keeping communication with the NC3R’s regional manager as well as with the Named Information Officer.

170. Investigating Cell Viability/Integrity In Vivo

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words
Cancer, Cell viability, Cell integrity, Genetic models, Therapy

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to use genetically engineered mouse models to understand how pre-cancer or cancer cells avoid dying and continue growing, leading to the development of cancer and, thereby, identify new treatment options for cancer therapy.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Work from this project will lead to new knowledge in fundamental cancer research and increase our understanding of how cancer begins, progresses and spread. It is likely that this will uncover potential new targets that are involved in cancer, leading to the

development of new treatments, which will be tested using mouse models of human cancer.

What outputs do you think you will see at the end of this project?

This project will provide new knowledge about the changes cancer cells undergo to avoid dying and continue growing, the components involved in causing these changes and how this leads to the development and spread of cancer. The knowledge gained will be shared through presentations at national/international conferences and publications in academic journals. New genetic mouse models, tissue specimens and cell lines may also be generated, which can be shared with other scientists to advance this field of research.

Who or what will benefit from these outputs, and how?

In the short term - Findings arising from projects in this licence will advance our knowledge in fundamental cancer research and increase our understanding of how cancer begins, progresses and spreads. These findings will be disseminated globally through open-access, peer-reviewed academic journals, national and international conferences, benefiting other scientists working in this field of research. Transgenic animals that are developed and characterised in this licence will also be valuable to other scientists interested in working with these mouse models.

In the medium to long term - By identifying potential new components in the human body that control the way cancer cells live, grow and spread, or engage the body's immune defence, we can proceed to test whether drugs that affect these components will kill cancer cells and prevent the spread of cancer in pre-clinical mouse models of cancer. Any positive findings can be communicated to clinical oncologists to establish collaborations for translation to the clinic. The identification and characterisation of potential anti-cancer targets in pre-clinical mouse models may lead to the development of new therapeutic agents for patient treatment and subsequent testing in clinical trials.

How will you look to maximise the outputs of this work?

In the short term, we will share the outputs of our work via internal meetings, seminars and workshops. Further dissemination to the scientific community will be through collaborations, where appropriate, and through presentations at external departmental seminars and research conferences. Ultimately, we plan to publish our findings from the work in peer-reviewed scientific journals. Once accepted, we will also communicate our findings via our website and Twitter.

Species and numbers of animals expected to be used

Mice: Mice: 40,000 mice over 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use mice for our studies due to their anatomical, physiological and genetic similarity to humans, which can provide insight into human physiology and disease. Mice are easy to handle and maintain, have short breeding and life cycles, and there is a vast amount of resources available for the study of mice. Most of the mice we will be using in the procedures for this project will be juveniles and adults, with a small proportion (<10%) aged up to a maximum of 2 years.

Typically, what will be done to an animal used in your project?

Approximately 70% of the animals will be used for breeding or generated from breeding and will not experience any adverse effects except for ear notching (for identification and genetic testing). Control or test groups of some genetically modified animals that arise from the breeding will develop or be predisposed to developing cancer. A smaller proportion of animals will develop tumours because cancer cells have been injected under the skin (implanted) and allowed to grow. Some of these animals will undergo irradiation (to damage DNA and cause cancer), be administered substances/agents (which will affect cancer development/progression, change aspects of their metabolism, alter components involved in cell death or growth, or be used for monitoring/analysis), therapeutic treatments, fasted or fed altered diet (eg. high fat diet) and/or alcohol.

Substances or treatments may be given through injections (subcutaneous, intraperitoneal, intravenous), by feeding in diet or drinking water, or through inhalation by the nose. The dose and frequency (eg. daily, 2-3 times a week) of administration of a substance/treatment will be dependent on the nature of the treatment itself, but will be based on extensive previous experience, published literature or information provided by drug companies. In some cases, animals may be subjected to multiple procedures during their lifetime (up to 8 procedures per animal, performed on separate occasions), repeated exposure of a single drug (eg. daily or 2-3 times a week dosing) or a combination of more than one agent/treatment. For multiple or combination treatment, dosing regimens will incorporate sufficient recovery periods between dosing to limit impact of the treatment on welfare of animals. For some procedures, anaesthesia will be used to minimise discomfort (eg. imaging).

Most procedures will be short term, between 1-6 months. Tumour development studies are normally between 3-12 months, with a small number of experimental and tumour cohorts (<10%) aged up to an absolute maximum of 2 years.

What are the expected impacts and/or adverse effects for the animals during your project?

Approximately 70% of the animals in this project that are used for breeding or generated from breeding will not experience any adverse effects except for ear notching (for identification and genetic testing).
These will be humanely killed when no longer required for breeding.

The remaining 30% of experimental animals may develop tumours, aged and/or undergo treatment/investigative procedures (eg. imaging, blood sampling), and may experience discomfort and develop clinical symptoms such as weight loss, abdominal swelling, hunching. Some animals may also develop model/disease-specific clinical signs such as papillomas (benign skin growths), jaundice and diarrhoea.

Most investigative and some treatment procedures will cause only transient discomfort with no lasting harm (<12 hours). However, other treatment procedures and tumour development may lead to longer discomfort (>12 hours) and up to moderate harm. Regular monitoring of animals will be carried out to avoid unnecessary animal suffering and to recognise when pre-determined humane endpoints are reached.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of animals used (~70%) will not have any observable clinical signs (sub-threshold severity). They are used for breeding programme only and will be humanely culled once their genetic statuses are known.

Those that develop signs will have mild (~10%) or moderate (~20%) severity symptoms.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We want to translate our findings obtained from cell cultures to the context of the whole animal, to obtain a closer and more accurate representation of human physiology and cancer. This is particularly important when we want to distinguish between the role/contribution of DNA mutations within the primary tumour and that of surrounding non-cancer tissue or immune cells during the development of cancer. It is only in the context of the living animal with the complexities of the immune system and surrounding tissue networks that we can fully understand how cancers grow, invade and spread.

Moreover, animal studies are instrumental for the discovery and study of cancer treatment options. They serve as essential pre-clinical models for understanding how cancer drugs are absorbed, distributed and eliminated from the human body, and importantly, for detecting unforeseen toxic effects.

Which non-animal alternatives did you consider for use in this project?

We use established cancer cell lines bought commercially or isolated from animal tissues in-house. We also use more advanced, 3-dimensional tissue culture systems (eg. spheroids or organoids) as well as mathematical modelling to simulate a solid tumour in the body.

Why were they not suitable?

Although some aspects of cancer research can be conducted using cancer cell lines and tissue cultures in the laboratory, the complexity of multiple genetic changes, accumulation of numerous pathological events and interplay between the primary tumour, microenvironment and immune system, can only be properly assessed within the context of the whole animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We recently secured substantial funding from CRUK for the next 5 years, which represents a continuation and expansion of our current work, and we also have an additional external grant for the next 2 years, both of which involve significant animal work. We estimated the numbers of animals used based on the numbers used at the peak of our current license and the fact that we have this increased level of funding for the forthcoming years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For experimental design, we are guided by the Experimental Design Assistant tool from the NC3Rs website. We use power calculations to determine appropriate experimental group sizes required for statistical significance.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We always perform pilot experiments with a few mice before scaling up for a full study, if promising observations are made. The ongoing monitoring of animals ensures experiments will be terminated as soon as sufficient data has been obtained, thereby minimising suffering and animal numbers. Non- invasive imaging techniques will allow monitoring of tumour growth/spread without unnecessarily culling of the animals. We use inbred strains when establishing cohorts to limit experimental variability and, therefore, limit the need for bigger cohort size. We often share common mouse strains with colleagues and freeze sperm from lines not immediately required to avoid unnecessary breeding. We also collaborate with other groups and share tissue or sera samples, whenever possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For this project, we will be using genetically modified (GM) mouse models or transplantation of modified cells into recipient mice. Whenever possible, we use the mildest approach to address our question – i.e. transplantation models rather than GM mice, as transplantation models are shorter term and involve less side effects. Most of our mouse models will only develop disease in a targeted organ, either upon administration of activating agents or if they carry the specific genetic alteration - thereby avoiding off-target or developmental effects (and therefore unnecessary suffering or harm).

Why can't you use animals that are less sentient?

Cancer is predominantly a disease of old age and the development and progression of the disease involves a dynamic interplay between cancer cells, the immune system, and surrounding non-cancer tissue. For these reasons, juvenile or adult mice (and a small proportion of aged mice) are the most suitable specimens for this study. Due to genetic and biological similarities, mouse models also better reflect the complexities of human cancer when compared to less sentient species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All experimental cohorts are monitored regularly and carefully, with studies stopped when statistically significant data is obtained, thereby ensuring minimal animal suffering without compromising on data quality. From the knowledge of previous experiments/literature, we avoid crossing mouse genotypes

that give rise to unnecessarily severe symptoms in the offspring. For therapeutic studies, we will use established and widely applied methods, such as diet manipulation (eg high fat diet) and drug treatments. For new treatment options, pilot studies will be carried out to establish drug safety/toxicity and careful monitoring will be undertaken. Our animal unit is proactive with environmental enrichment (e.g. chewing block, nesting material, gel diet supplementation) and the use of anaesthesia and analgesia under guidance from the NVS is routine practice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For all our studies, we will refer to the Guidelines for the welfare and use of animals in cancer research (Workman et al, 2010 and in its revised form, when published) and ensure best working practice. We will consult the NC3Rs guidelines and monitor refinement where such practices are published (NC3Rs/LASA)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep abreast of advances via the NC3Rs and LASA websites. In addition, developments are disseminated via Institute-wide forums for all animal users. Similarly, any changes to procedure resulting from advances in the 3Rs will be considered and

where appropriate, implemented on an Institute-wide basis. We will also take continual advice from the NACWO, NVS and NTCO.

171. Combination Immunotherapy for the Treatment of Cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  -

Key words

Cancer, Immunotherapy, Immunology, Radiotherapy, Combination therapy

Animal types	Life stages
Mice	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The main aim of this work is to increase our understanding of the immune response to cancer and develop new treatments that will enable the immune system to function more effectively in eliminating cancer cells

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Evidence from pre-clinical and clinical studies demonstrates that the immune system can contribute to the control of cancer. Standard of care treatments such as radio- and chemo-therapy are now understood to work at least in part through modulation of the immune system, and therapies designed to promote cancer immunity (immunotherapy) have revolutionised the way in which cancer is treated in the clinic and greatly improved

outcome for many cancer patients. However, we are still a long way from fully understanding how to best exploit the immune system to achieve effective cancer control. Immune evasion is a key hallmark of cancer, and many patients fail to respond, or develop resistance, to even the most effective immunotherapies, such as checkpoint inhibitors. These are a type of immunotherapy that can be used to help boost the activity of white blood cells (such as T cells) that are able to recognise and kill cancer cells, but are often prevented from doing so due to suppression by immune checkpoints. This project aims to: increase our understanding of how the immune system contributes to the control of cancer following anti-cancer therapy; identify mechanisms of immune activation and suppression that may impact on this response; and use this information to develop more effective combination approaches, that will improve outcome through the generation of potent anti-cancer immune responses. The aim of this proposal therefore is to enhance scientific knowledge through discovery research that we hope will lead to the development of more successful and effective anti-cancer therapies and eventually, greater benefit to cancer patients.

What outputs do you think you will see at the end of this project?

The output of the research will lead to advancements in scientific knowledge and will potentially contribute to the development of new therapeutic approaches for the treatment of cancer. In particular, we will increase understanding of how we can combine immunotherapy with standard-of-care treatments, such as radio-therapy, or other novel therapeutic approaches, to enhance immune responses to cancer. This work will involve:

- Development of pre-clinical cancer radio-therapy and new tumour models that are more representative of human disease.

- Evaluation of the impact of anti-cancer therapy on immune responses in tumour and normal tissue.

- Discovery research into key therapeutic targets that may enhance the generation of immune responses to cancer.

- Therapeutic evaluation of new treatment strategies and associated mechanisms of action.

This information will be shared with other researchers in the field and the broader scientific community through a variety of outlets including publication in peer-reviewed journals and presentation at National and International conferences. Research will be shared with the wider public by making our publications open access, sharing key findings on social media and web-based platforms, and via outreach , for example, at patient and public engagement events. Our work has direct clinical application which will be pursued through collaboration with clinicians based locally and nationally across the UK, who will provide translational perspective and enable evaluation in early phase clinical trials.

Who or what will benefit from these outputs, and how?

Using mouse models of cancer we will seek to:

1. Understand the impact of anti-cancer therapy on local and systemic immune responses in tumour and normal tissue. This information will be used to guide the

rationalised development of new therapeutic combination approaches with the aim of augmenting cancer immunity (short-term).
2. Evaluate the therapeutic benefit of novel therapeutic approaches and explore the mechanistic basis for treatment response. This will identify potential new treatments for cancer and begin to inform our ability to stratify patients most likely to benefit from immunotherapeutic approaches (mid-term).

3. Identify factors in the area surrounding the cancer cells (the tumour microenvironment) and other factors outside of the tumour that may inhibit anti-cancer immune activity. This discovery science will increase knowledge of immuno-regulatory pathways within the tumour microenvironment and lead to the identification of new therapeutic targets that may further enhance outcome (mid-term).

Thus, data generated from these studies will benefit the scientific community through shared knowledge, enhancing collective understanding and facilitating collaborative and independent research advances.

4. Data obtained from work conducted under this license will be used to guide the development and patient stratification in investigator-led clinical trials, run in multiple centres across the UK. We have a track record in establishing trials and translational immune monitoring based on previous pre-clinical science within the group (under our previous Project Licence) (long-term).

Thus, work conducted under this licence will benefit cancer patients through the development and translation of more effective therapeutic approaches.

5. Key findings from research conducted under this license will also be included in lectures and other teaching related activities in order to enhance educational learning through cutting-edge, research led teaching (mid-long term).

Thus, this work will benefit the next generation of cancer researchers and clinicians enhancing awareness and knowledge through education.

How will you look to maximise the outputs of this work?

Our findings will be made available to other scientists through collaborations, publication in high profile peer-reviewed journals and presentations at scientific conferences and meetings. Our Establishment has a policy of ensuring that all publications generated are available on open access to all. In addition, our work has direct translational and clinical applications that we will investigate through collaborations with clinicians at the Establishment and other medical cancer centres worldwide. Data will also be shared with the general public through outreach activities within the local community, social media and other public engagement activities.

Species and numbers of animals expected to be used

- Mice: 11000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Immune responses to cancer are highly complex, dynamic and involve cross-talk between many different cell types, organs and tissues. The progression of an immune response in a host organism involves the coordinated interaction of multiple integrated signals that evolve over time in a 3D- environment. Similarly, cancer development and progression involves numerous interactions between tumour and host immune cells. These responses can be influenced by both local microenvironmental signals within the tumour and by systemic cues, including from distant organs. These factors, combined with the involvement of multiple host cell-types and the expansion and migration of rare tumour-specific effector cells, mean such research cannot be carried out in tissue culture alone or reproduced using experiments simulated on a computer (in silico) and can only be addressed with the use of animals.

The mouse is an ideal organism in which to study immune responses to cancer. The mouse genetic code is of a similar size to the human genome, with a great deal of functional conservation between the two species, and there is considerable similarity between gene expression profiles in the mouse and human immune system. Whilst mouse biology mirrors that of the human remarkably well, immunological differences between the two are increasingly well characterised and understood (for example mapping the mouse immunogram is an on-going collaborative project with data shared in a public repository available at http://www.immgen.org/) allowing meaningful interpretation to be made. An extensive catalogue of genetic mutants exists, facilitating the exploration of immune function including transgenic knock-in and knock-out mouse models. Experiments demonstrating that new types of therapy work in mice have led to the development of successful new treatments for cancer

patients. A number of treatments which can help boost the immune response to cancer including immune checkpoint antibodies and chimeric antigen receptor (CAR) T-cells (T cells which have been modified in culture to make them better able to attack cancer cells) have been developed using studies in mice. These have been very effective in cancer patients and changed the way that many Doctors treat cancer. Thus, mice remain an important and highly relevant research model.

This proposal will use adult mice. The majority of studies will utilise young adult mice (typically 8-12w), although studies of aged-immune responses may use elderly mice (up to 18 months of age at the time when the experiment is started; equivalent to elderly humans, ~60-70y). Aged mice better represent the aged human population that typically develop cancer. This is important as the immune system is known to enter functional decline with age which may limit the immune response to cancer and treatment efficacy.

Typically, what will be done to an animal used in your project?

Mice may have tumours implanted at heterotopic sites (e.g. subcutaneously on the flank) or orthotopically (e.g. in the mammary fat pad for breast cancer). Tumour growth is generally not associated with pain during the period in which we conduct our observations. However, on occasions some of the tumour growths may become ulcerated. Under these circumstances we regularly monitor their progression very carefully, and manage their treatment to minimise the potential pain or discomfort, taking advice from veterinarians and highly experienced animal technicians. The growth of tumours will be assessed by either imaging with ultrasound or when possible, such as in the case of subcutaneous or intradermal tumours, using callipers. Fast growing tumours will be monitored daily.

Some mice may have either potential novel therapeutic agents, existing clinical agents or placebo administered by a variety of routes, but usually either orally, or by injection either under the skin, into the abdomen or into the tumour to study the effects on tumour growth and / or tumour composition. Some mice will receive radiotherapy to replace their immune system with specific populations of blood cells from donor mice or to treat their tumours. The mice will also have blood samples taken either from the tail vein whilst conscious or by sampling from a heart chamber under terminal anaesthesia (in which case the animal does not regain consciousness before humane termination).

Some mice will have core needle biopsies taken from their tumours; others may undergo surgery, for primary tumour removal, and these will be anaesthetised for the operation and receive pain killer post- operatively to manage any pain they experience. Some mice will also have repeated anaesthesia for the purposes of imaging the internal tumours. Whilst loss of consciousness may be distressing, this is not painful.

Mice may be used up to the age of 18 months at the time the experiment is started in order to study the effectiveness of treatments on tumour growth in older mice that better represent elderly human patients. Some mice may also be given altered diet that will change the way in which tumours grow or the immune system responds. This diet will be nutritionally balanced so will not impact the general welfare of the mouse.

Mice will be group housed in ventilated cages which have their environment enhanced with items such as tunnels, houses, nesting material and gnawing blocks.

At the end of any protocol mice will be killed humanely.

What are the expected impacts and/or adverse effects for the animals during your project?

Tumour growth is generally not associated with pain during the period in which we conduct our observations. However, on occasions some of the growths may become ulcerated or interfere with bodily functions. Metastatic tumour effects are anticipated to be sub-clinical but mice will be monitored carefully for signs of pain and discomfort throughout the course of the experiment. The models we use mostly metastasise to the lungs indicated by shortness of breath or trouble with walking due to breathlessness

The vast majority of the procedures will result in no more than transient discomfort (typically up to ~72 hours) and no lasting harm. Injections would only cause very transient pain. Some mice, especially those that receive the most effective therapies, and/or therapies involving radiation, show transient weight loss (for a few days following treatment). After any surgical procedures we will monitor mice for signs of pain and administer effective pain relief for as long as it is required.

Any mice unexpectedly found dead overnight when monitoring indicates that they are normal will be reported to the HO. This is most likely due to a sudden impact of the spread of cancer cells from the original location to other body organs. The health of all mice will be observed daily. Notwithstanding this, all the mice will be humanely culled at the end point of the experiments.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The vast majority of mice are only expected to experience mild to moderate clinical symptoms due to tumour growth before they are humanely killed. A number will experience ulceration of the tumour growth. The number of animals mice that experience ulceration varies depending on strain, and tumour type. Where ulceration occurs this will managed to moderate severity. We have extensive experience in our center and on our previous licence. Some mice will experience the discomfort of repeated (daily) injections of therapeutic agents or oral delivery. We will aim to utilise the least stressful route of administration wherever possible.

Mice that undergo surgery will be anaesthetised for the operation and receive pain relief peri- operatively until pain subsides. Some mice will also have repeated anaesthesia for the purposes of imaging the internal tumours. Whilst loss of consciousness may be distressing this is not painful.

Based on our experience over the course of the previous licence, our anticipated severity proportions for this project are:

50-60% mice mild (mice undergoing simple non-surgical subcutaneous implantation of tumours that do not grow beyond a certain size and do not ulcerate); 40-50% moderate (mice bearing more invasive tumours that may ulcerate and undergo surgical procedures). We aim to minimise the appearance of severe endpoints with careful monitoring, surveillance and care. The actual severity varies across specific studies so these values are averages based on the previous 5 years annual returns, for all mice used.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The development of effective anti-cancer therapies is a major goal for modern biomedical science, and the immune system has great potential for contributing to the control and elimination of malignant disease. Therefore, therapeutic strategies to target the immune system against cancer are an attractive proposition. This project aims to develop and characterise therapeutic combinations designed to enhance anti-cancer immune responses. However, such therapies involve complex interaction between tumour cells, constituents of the tumour microenvironment and components of the host immune system. These dynamic interactions occur between numerous cellular populations, in different tissues and organs including the tumour itself, and due to the nature of their complexity, they cannot yet be fully reproduced in silico or in vitro. Consequently, it is currently impossible to fully recapitulate the phases of a therapeutic anti-cancer immune response, homing to the site of tumour, and associated immune checkpoints/regulation, outside of a living organism. Mouse models represent an ideal system for studying these interactions.

The mouse is a model organism that closely resembles humans. The human and mouse genomes are approximately the same size, and display an equivalent number of genes, which are functionally conserved. Definitively, mouse models are important for placing the findings of in vitro studies or correlative analysis of human samples into an appropriate and meaningful in vivo (studies performed in a living organism) context. It is the combination of in vitro (studies performed using cells cultured in a lab) and in vivo studies that provides the insight needed to understand cancer biology and develop new therapeutic approaches, and there are no effective approaches to hand that can replace the in vivo studies, as these allow the in vitro findings to be tested in an appropriate environment.

Which non-animal alternatives did you consider for use in this project?

Caenorhabditis elegans, i.e. nematode worm, and Drosophila melanogaster i.e. fruit fly. Additionally, use of mice will be minimised, where possible, by using in vitro , ex vivo and in silico model systems. Moreover, we will continue to assess new ex vivo model systems to compliment the in vitro models.
We will continue to test and develop these in vitro and ex vivo systems over the next few years to address how well they can effectively model immune responses to cancer.

Generation of organoids or explants will be considered and used as an alternative approach to assess responses where feasible. This would mean that a single tumour (from a single animal) could be used to investigate a number of therapies and reduce the number of mice involved in the study.

Why were they not suitable?

The study of cells in culture (in vitro) and less sensitive organisms provides us with clues on the mechanisms of cellular processes in a simple and valuable context, which allows the establishment of hypotheses regarding the function of cells in a living animal. Whilst enormous progress has been made in the field of cancer research using in vitro models, there are a number of questions related to immune-oncology that can only be addressed using animal models of disease. In particular the heterogeneity, complexity, dynamics and whole organism level regulation of the immune response to cancer cannot currently be effectively modelled outside of a living organism. Thus, in order to investigate the relationship between the immune system, cancer progression and response to anti- cancer therapy, we need to perform studies in living organisms with an immune system that functions similarly to that of humans. The immune systems of lower order organisms such as C.elegans and D.melanogaster are too divergent from humans and frequently lack key elements such as adaptive immune cells that are critical for anti-cancer responses in humans.

Explant models have currently not developed to the point where they are comparable to animal studies for immune therapy, although this field is rapidly advancing, and we will follow this progression and consider these as alternative approaches where possible.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of mice to be used is estimated based on the following:

- We currently have 3 major projects as well as a number of PhD studentships that will directly interrogate the interactions between the immune system, cancer and anti-cancer therapies. These, all have planned in vivo studies, and rely on in vivo models to test specific hypotheses generated from in vitro studies.

- We anticipate that new studies will start and develop over the next 5 years which will require extensive analysis of tumour/immune interactions and effect of therapeutic intervention, which will require in vivo modelling.

- From our experience using “real world” data based on the number of mice used in previous years, including over the last 5 years during the course of the previous licence. This has typically ranged between 1500-2600 mice per year. Group sizes of between 5 and 8 mice (dependent on the readout) per experimental group suffice. We would typically examine more than one model subpopulation/line. Likewise, we may use several doses of a drug, or several different drugs or treatment combinations to test a hypothesis.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The use of mice will be minimised in several ways:

- By considering on-going statistical estimation of power requirements in each of the studies, using prior results in order to use the minimum number of mice while retaining sufficient numbers for statistical significance. In general, we will use a sample size capable of detecting a 50% practical difference with 80% power and 95% confidence.

- By incorporating as many test groups as possible within a single controlled experiment, reducing the number of controls required compared to a series of smaller experiments.

- By utilising tissues from different sites on one mouse for both treatment and control samples.

- Experiments will be designed using the principles outlined in the experimental design tool on https://eda.nc3rs.org.uk/ (such as randomisation, power, blinding) and reported following the ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- By doing as much preliminary work as possible in culture models in vitro and in silico analysis prior to engaging in in vivo studies.

- By minimising variability in results through utilising inbred strains and by housing them under identical conditions to limit variability.

- By performing pilot studies using small numbers of mice when information is lacking in the literature/from collaborators, so that the number of mice utilised in experiments is reduced to minimal levels.

- By using optimal breeding strategies to reduce the number of genetically engineered mice.

- By running experiments in parallel so that they can share a single control arm where possible

- By taking care to ensure that each experiment is appropriately analysed and that the maximum amount of information is gathered thus reducing the need for experiments to be repeated.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The use of inbred and fully back-crossed mice not only reduces intragroup experimental variability but also eliminates incompatibility when cell transfers are carried out between various knockout, transgenic and wild-type strains.

We will continue to refine experimental protocols wherever possible, to minimize both the number of mice used and their suffering. Throughout experiments, we will monitor tissue and tumour growth and the health status of transplanted mice with pre-defined frequencies. Any mice that have health related issues related to surgery, transplantation or experimental therapy will be given immediate attention with a view to alleviating symptoms or discomfort and will be killed humanely if adverse effects are considered too severe to be treated.

Some of the models/treatments may lead to tumour ulceration. This will be carefully monitored and stop/go decisions made according to a "traffic light" system that uses a grading approach based on size and appearance of the ulcer. Briefly, Grade 1 ulcers, <0.5cm, presented as red colouration, cracking or abrasion of skin, will receive emollient cream and remain on study; Grade 2 ulcers, 0.5 - 1cm, skin presenting as dry, scabbed, slight clear exudate, or <0.5cm with full thickness defect, will be assessed and treated for pain as required, re-graded daily, and remain on study; Grade 3 ulcers,
>0.5cm with cloudy exudate, pus, bleeding or mouse showing clinical signs of moderate severity, or
>1cm, mouse will be humanely killed.

Why can’t you use animals that are less sentient?

The mouse cancer models that we will use very closely recapitulate human disease and thus allow us to understand the molecular and cellular events and steps involved in the activation of tumour immunity in response to cancer progression and anti-cancer therapy. The mouse is far more similar to humans than other less sentient animals and this is critical for increasing our understanding of cancer immunity and developing therapies that can be translated to the clinic.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We constantly work to improve husbandry and procedures to minimise actual or potential pain, suffering, distress or lasting harm and/or improve animal welfare. Animals will be groups housed wherever possible, provided with enrichment and handled by either tunnel of cup-handling. Animal suffering will be minimised by making every effort to keep the tumour models employed at the subclinical levels. Wherever possible, this will be achieved by using non-invasive imaging modalities to monitor tumour growth and the development of metastatic disease. In addition, as detailed in the individual protocols, steps will be taken to minimise the severity of the procedures. Finally, we will ensure that all mice receive the highest standard of care, and preventative medicine (including anaesthesia and analgaesia where required) will be used.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Surgical procedures will be carried according to the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery.

Relevant published literature will be used as template for experimental design and decision making (Workman et al., 2010. Guidelines for the welfare and use of animals in cancer research. BJC, 102, 1555-1577).

We will follow guidelines of good practice [Morton et al., Lab Animals, 35(1): 1-41 (2001); Workman P, el al. British Journal of Cancer, 102:1555-77 (2010)]. Administration of substances and withdrawal of blood will be undertaken using a combination of volumes, routes and frequencies that themselves will result in no more than transient discomfort and no lasting harm.

Where we use aged mice we will refer to Wilkinson (Laboratory Animals 2020, volume 5(34)), relating to the husbandry and care of aging mice.

We will consult the NC3Rs guidelines and monitor refinement where such practices are published (NC3Rs website and elsewhere).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By reading 3Rs literature and participating in 3Rs workshops locally and nationally. Through discussing refinements with our NACWO, NVS and ASRU. I am a member of the Establishment AWERB and regular attendee and contributor to our Retrospective Review and Licensees meetings and the 3Rs Poster session, all of which take place annually at our Establishment.

172. Biocompatibility of a Prototype Fully Implantable Auditory Implant Microphone

Project duration

2 years 0 months

Project purpose

- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Deafness, Hearing rehabilitation, Microphone, Biocompatibility, Cochlear implantation

Animal types	Life stages
Guinea pigs	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To assess whether or not it is possible to successfully implant a functioning microphone into the ear of guinea pigs without it being rejected. This will eventually be incorporated into a fully implantable cochlear implant that will overcome the issues arising from current cochlear implant technologies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The World Health Organisation estimates that 430 million people globally have disabling deafness. This amounts to over 5% of the world's population. It is estimated that this will increase to 25% by 2050. Milder forms of deafness can be managed through amplification of sound using hearing aids but profound hearing loss requires cochlear implantation to rehabilitate hearing.

Cochlear implants are electrical devices that consist of two components, an implantable component that consists of an electronic s package and an electrode array that is placed inside the cochlea and a sound processor that picks up sounds, converts them to an electrical signal and transfers the signal by radio to the implanted component. The electrodes in the array are then stimulated and this activates the hearing nerve which sends a signal to the hearing centres of the brain providing the perception of sound.

Whilst cochlear implants are a very effective way of restoring hearing to those with profound deafness, the external sound processor has several disadvantages. It is not well accepted cosmetically as it is quite bulky. It is inconvenient to have to wear the processor all the time. It can be inconvenient during exercise, outdoor sport activities and swimming. It would therefore be desirable to develop a fully implantable cochlear implant.

There have been several attempts to produce auditory implants with a microphone that can be implanted in or around the ear, thus avoiding an external component. Although these look better and are more convenient, those that have been developed to date have significant limitations including loss of signal because of implantation under the scalp, picking up body noises, positioning away from the ear, and bulky size making implantation challenging and prone to complications.

This project aims to develop a fully implantable microphone that addresses the limitations set out above and can be integrated in to existing auditory implant technology as well as preserving existing hearing. This is important because it will make cochlear implants more practical and more effective.

There is a great deal of development work required to produce a microphone that meets these requirements. In particular, it is important to know if the microphone is stable in the implantation site and whether it is able to effectively pick up sounds. It is also important to develop a safe, effective and quick surgical technique for implantation and to ensure that the microphone is not rejected by the body. It is not practical to carry out this development work in humans without risking significant complications and/or device failure. It is therefore important to use an animal model prior to transitioning to human implantation and as such this study will provide proof of concept.

What outputs do you think you will see at the end of this project?

The expected end result of this project is a fully implantable microphone that:

1) Can be safely, quickly and easily implanted

2) Is able to pick up sounds effectively

3) Does not cause any tissue rejection and is stable in its position

4) Does not detrimentally impact on the animal's existing hearing

Who or what will benefit from these outputs, and how?

Hearing impaired patients who receive auditory implants will benefit from this device. The device will improve the hearing and quality of life of the recipient through:
1) Better sound quality: There will be less sound attenuation because of the thin tissues overlying the implant. This will have positive social and professional implications

2) Better cosmesis: The implant will be fully implantable with no visible external components. This will make the implants more widely acceptable without the stigma of wearing a visually prominent sound processor.

3) Easier implantation than existing implantable microphone designs: The microphone will be much smaller than the existing devices available and can be implanted easily within the same surgical field as existing cochlear implants

4) Reduced body noise: Its location within the ear canal means that the microphone is not prone to body noises such as rustling of the hair

5) More natural positioning of the microphone: Its location within the ear canal means that sounds are picked up from the same location that they would be in normally hearing individuals. This will make sound localisation easier and will make the use of a telephone more natural. It may also enable use of headphones.

6) Reduced energy requirements and therefore better battery life: Its small size and low energy requirements mean that it does not drain the battery of the device as much as the current generation of implantable microphone

7) A wider range of environments in which the implant can be used: The implant can be on all the time no matter what environment the implantee is in. This has significant safety and security

implications. For example, it can function whilst sleeping so that a mother can hear her child; a child can swim whilst the implant is active and therefore hear what their parents are saying; the implant can be used whilst wearing protective head gear eg. Crash helmets, riding hats

8) Activities of daily living will be easier: The device could be conveniently used in the shower or whilst bathing

9) There will be no complications from wearing an external processor, in particular, avoiding irritation of the skin associated with it and avoiding the risk of losing or damaging the processor should it become dislodged.

There are also other potential scientific benefits from the project. It will improve our understanding of the biocompatibility of the materials used in implants and the nature of the host's inflammatory response to the materials used. It is hoped that this will be of benefit to scientists developing and designing implantable materials (not just in the field of auditory implantation) and to the commercial manufacturers of such devices.

How will you look to maximise the outputs of this work?

The results of the project would be distributed nationally and internationally via presentation in conferences and publications.

Once the aims set out above are achieved, the microphones can be incorporated in to existing auditory implants in collaboration with implant manufacturers.

Species and numbers of animals expected to be used

- Guinea pigs: 9

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

For this project adult guinea pigs will be used. Adult guinea pigs have an ear drum that is a similar size to the human ear drum. They also have an ear canal that is close enough to the human ear canal to make it appropriate for the purposes of this project. Guinea pigs are a common model used for research related to the ear and there is extensive literature demonstrating that they are a good model in projects similar to this one.

Typically, what will be done to an animal used in your project?

Approximately 9 animals will be required initially for this study.

1) The animals will undergo general anaesthesia(GA)

2) The animals will undergo a hearing assessment whilst asleep, prior to implantation to ensure that they have normal hearing. This will involve measuring the brain activity in the hearing centres of the brain in response to sound.

3) The animal will be implanted with a microphone. This will involve surgically accessing the ear and placing the microphone in the wall of the external ear canal, covered with ear canal skin.

4) A wire from the microphone will be extended through the incision in the skin through which the implantation has been performed. This will enable testing of the effectiveness of the microphone once it is in position

5) The animals hearing will be assessed again to ensure that it has not been damaged

6) The animal will be humanely killed at defined time points and the microphone and the tissues around it will be assessed for inflammation to determine the stability of the microphone in its implanted location. This will involve analysis of processed tissues under the microscope.

What are the expected impacts and/or adverse effects for the animals during your project?

After surgery the animals will experience minimal pain for 2-3 days. This will be controlled with simple analgesics. In uncomplicated cases the ear should be fully healed within a week and there should be no adverse effects on the animal. There is, however, a risk of adverse reaction to the general anaesthetic and guinea pigs are known to be more susceptible to this than other animals. Careful anaesthesia will minimise this risk.

There is a risk of adverse reaction to the general anaesthetic, mainly from respiratory depression. From the experience of other research teams, up to 10% of animals may be

affected by this. Every effort will be made to minimise this through good quality anaesthesia.
Potential surgical complications include wound infection, infection at the site of implantation, reduced hearing and extrusion of the microphone housing.

The wire extending from the microphone through the wound could be irritating to the animal and if so, this will be cut flush with the skin and the wound allowed to fully heal. This will mean that subsequent testing of the function of the microphone will not be possible but the measurements taken during the initial surgery should be adequate to determine if the ability of the microphone to pick up sounds is adequate.

The implanted animals will be humanly killed at defined time points (either 6 week, 3 months and 6 months after implantation).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The surgical procedure is regraded as moderate in severity. All animals will be exposed to the same procedures.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of this project is to assess the tissue reaction after a certain period of exposure to the prototype microphone. The animal experiments are required in order to assess the effect on living tissue before being trialled in humans. This will ensure that the microphone is not rejected. Guinea pig ear anatomy resembles human ear anatomy which helps in the development of the surgical technique for implantation and allows assessment of the ideal position of the microphone within the ear. It will also give provisional information on the tissue reaction produced by the presence of the microphone ie. degree of rejection, if any, prior to implantation in humans.

Which non-animal alternatives did you consider for use in this project?

Cadaver dissection to develop surgical technique and siting of the microphone prior to commencement of live animal work will be used to minimise the learning curve on the live animals.

Laboratory based tissue culture experiments are also being undertaken to assess if there is a tissue reaction produced by the materials within the microphone. This will ensure that

any adverse reactions on a cellular level will be identified prior to implantation in animals and if present these materials will be excluded from the microphone.

The alternative to using a guinea pig model is to use a different animal model or to use human experiments from the outset. There are no other non-animal models that could be used in this project.

Why were they not suitable?

The anatomy of other animal models differs from human anatomy to a greater extent and these models are therefore less suitable. It is inappropriate to go straight to human experiments given the uncertainty around the types of tissue reaction generated by implantation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Statistical advice has been used to help determine the number of animals required.

It is necessary to assess the tissue reaction to the implant at different time points (6 weeks, 3 months and possibly 6 months). In order to have an understanding of the range of tissue reactions that might occur, and to allow for some anticipated deaths amongst the animals during the study, we would like to implant 6 ears at each time point. This makes a total of 18 ears in 9 animals.

An initial cohort of 3 animals will be implanted bilaterally and then humanely killed and examined at 6 weeks. If there is no evidence of any tissue reaction at that point then a further 3 animals will be implanted bilaterally and humanely killed and examined at 3 months. Again, if there is no tissue reaction then a final 3 animals will be implanted bilaterally and humanely killed and examined at 6 months.

If at any time point there is evidence of a tissue reaction or other issues with the device then the design of the device will be modified to address the issues and a further cohort of 3 animals will be implanted bilaterally and the process repeated. Depending on the severity of the issues that arises, minor modifications will be made or wholesale redesign will be performed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We had detailed discussions with a statistician to reduce the number of animals required as much as possible. The initial 6 week trial will identify any early problems with the device and enable modification as required without using excessive numbers of animals. The stepwise increase in duration of implantation in small groups of animals provides us with

the opportunity to determine any issues that might arise with the minimum number of animals.

We will be developing and refining the surgical technique for implantation on cadaveric animals prior to commencement of implantation in live animals so that the risk of complications arising from technical failure is minimised.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The initial 6 week cohort will identify any early issues that might arise from implantation and will allow modification of the protocol, as required, for the longer duration of follow up. This will minimise complications and animal deaths in the subsequent, longer follow up, groups. We have reduced the number of animals in each follow up group as much as possible to allow assessment of the tissue reaction to the microphone whilst allowing for unexpected animal deaths.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Adult male guinea pigs will be used during this project. The use of a single sex simplifies the care of the animals operationally.

These animals will be anaesthetised prior to implantation and standard aseptic techniques will be used during surgical procedure. This will prevent any discomfort during the implantation procedure and will minimise the risks of post-operative infection.

The study design has been refined in collaboration with experts in the field. All procedures will be performed by an appropriately trained person. The initial experiments will be undertaken under the supervision of someone experienced in undertaking guinea pig experiments. The NTCO will also be present during the initial experiments to ensure competent conduct.

Postoperatively they will experience minimal pain which will be controlled with adequate analgesia. In recovery, animals will be closely monitored for signs of hypothermia, pain and distress.

Why can’t you use animals that are less sentient?

The ear anatomy of the animal needs to be as similar to the human ear as possible in order to provide as relevant a result as possible. The adult guinea pig ear is closest matched animal model to the human ear.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

During the surgical procedures adequate anaesthesia, analgesia and monitoring will be used as advised by the NVS to minimise potential harms to the animals. The staff undertaking the anaesthetic will be appropriately trained and experienced. Pain killers will be given following surgery. Following the surgery and during the follow up period, high standards of animal husbandry will be maintained. All efforts will be made to alleviate any distress and any signs of an inflammatory reaction/infection will be treated with antibiotics and anti-inflammatory medication. If this is not successful and the animal continues to show signs of uncontrolled distress then the animal will be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Laboratory Animal Science Association (LASA) guidelines will be followed before, during and after the implantation in order to maintain wellbeing of the animals. Peer reviewed publications involving similar experiments will be reviewed to ensure that the experiments are undertaken in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

ASPA guidelines will be strictly followed and the research team will regularly monitor the N3CR website. In addition, the research team will be in close contact with other researchers involved in animal work and any changes to recommendations around the 3Rs will be discussed. Similarly, the research team will be in regular contact with the NTCO and NACWO to ensure that any potential changes are identified promptly. The implementation of any changes will be in discussion with the NTCO and NACWO.

173. Nociception in an Animal Model of Parkinson's Disease

Project duration

5 years 0 months

Project purpose

- Basic research

Translational or applied research with one of the following aims:

  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words
Pain, Parkinson's disease, Rodent models

Animal types	Life stages
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to determine the parts of the brain that go wrong and lead to certain pain symptoms in Parkinson's disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Pain is a very common and troublesome, but poorly understood symptom in Parkinson’s disease. The project is focused on one particular type of pain in Parkinson's disease - central neuropathic pain.
Central neuropathic pain is often described by patients as otherwise unexplained painful sensations with no apparent origin, predominantly on the more affected side. It is thought to originate from changes in the function of the central nervous system as a result of the disorder, suggesting that the brain circuits that allow us to perceive and process pain could

function incorrectly in Parkinson’s disease. While abnormal pain processing has been reported in some brain structures involved in pain, there is to date no clear mechanism to explain the specific symptoms of central neuropathic pain.
Based on our recent discovery of a previously unreported network in the brain connected with pain processing, we propose to test a new hypothesis linking central neuropathic pain symptoms to abnormal activity of this network. This network includes brain structures in a collection of nuclei called the basal ganglia, which are known to be affected in Parkinson’s disease, and a primary pain processing structure within the brainstem called the parabrachial nucleus.

What outputs do you think you will see at the end of this project?

Although we already have demonstrated that the novel pain processing circuit we are investigating is activated by painful stimuli, this has been achieved by recording the activity of neurons in each structure separately. However, we are looking at the function of a circuit and the technique we are going to use - functional magnetic resonance imaging - will allow us to examine that circuit in its entirety, recording the activity of the constituent brain structures at the same time. Again, although we have shown that activity in the circuit is changed following lesions that mimic those occurring in the brains of people with Parkinson's disease, we have not been able to simultaneously evidence that across the circuit. From examining circuit activity, it will hopefully be possible to determine which structures in the circuit drive the abnormal activity and which simply follow their lead.

Who or what will benefit from these outputs, and how?

Around 85% of patients with Parkinson's disease are affected by some form of pain, and the origins of pain symptoms in the disorder are not understood. The results of this research are likely to provide new information on the central nervous system mechanisms and pathways of pain in Parkinson’s

disease which will be of interest to research scientists. In the longer term, it is hoped that by elucidating the circuitry involved in Parkinson's pain, new treatments can be developed that take that circuitry into account.

How will you look to maximise the outputs of this work?

The project is being conducted alongside colleagues in France and as a consequence the work will be known by and be available to relevant individuals on both sides of the channel very early on. Beyond that, and our general intention to make the data widely and freely available, we will endeavour to publish the results at the earliest opportunity. Given that particular attention will be afforded to experimental design, even negative results will be publishable. We also have funding to attend key conferences to disseminate the results of our research.

Species and numbers of animals expected to be used

- Rats: 250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The rodent basal ganglia and related structures have been widely studied and an extensive literature exists on all aspects of their structure and function. This and the fact that the structures are highly conserved in the vertebrate brain means our results will generalise across many species, including humans. The use of adult animals reflects that fact that Parkinson's disease is a disorder of adulthood.

Typically, what will be done to an animal used in your project?

All animals will be prepared for imaging and the application of stimuli, in particular electrical stimulation of (for example) the forepaws to assess how more intense stimuli are processed. Preparation for imaging will involve the administration of a general anaesthetic, cannulation of blood vessels for the administration of substances as necessary, such as additional anaesthetic, and for monitoring blood pressure and heart rate. Breathing will be assisted using a ventilator attached to the animal via a tracheotomy tube. Animals will then spend around two hours inside the magnetic resonance imaging machine being scanned. Prior to the imaging session, some animals will undergo surgery under recovery anaesthetic to induce small lesions of the dopamine neurotransmitter system that degenerates in Parkinson's disease, and in a subset of cases animals will receive an additional lesion of the subthalamic nucleus, a site that is known to be problematic in Parkinson's disease. Where the procedure includes anaesthesia, animals will be carefully monitored for adequate anaesthetic depth during the procedure and (where relevant) for adverse effects during recovery. If any procedural complications do arise, veterinary advice will be sought immediately from the named veterinary surgeon. In all cases the animals will be killed at the end of the experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

For the lesions affecting the dopamine systems, recovering animals will have a mild postural bias towards the lesion side and rotate in that direction when touched. Although there will be some initial weight loss after surgery (<10%), the ability to feed, drink and groom will not be impacted. Post-surgery complications due to this type of lesion are very rare (<2%). Any animals showing negative effects of the lesions, manifest as collapse or a hunched posture with their fur standing on end, or significant (>15%) weight loss, will be humanely killed. For the scanning, a suitable long-term anaesthetic protocol will be selected. Physiological signals such as heart rate, blood pressure and body temperature will be monitored throughout the experimental procedures for any indication of lightness of anaesthesia.
Observations for movement will be made throughout the procedures and the common reflex tests (e.g. hindpaw withdrawal) will be repeated at regular intervals to ensure depth of anaesthesia is suitably maintained. Supplemental doses of anaesthetic will be administered as necessary.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected level of severity for the recovery experiments is moderate, and brain imaging will be conducted under terminal anaesthesia. Following our initial work to image the circuit in unlesioned animals, all subsequent animals (90% of the requested number) will undergo recovery surgery.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This is a systems-level neuroscience project that will investigate a fundamental aspect of brain function. At present there is no alternative to using invasive experiments with animals to gain the knowledge required. However, the licence holder has 18 years' experience of working in close collaboration with computational neuroscientists who use biological data derived from our work to parameterise their computational models. The progressive refinement of these models will gradually replace the need for biological testing.

Which non-animal alternatives did you consider for use in this project?

We have thoroughly considered non-animal alternatives, and very much hope that our future computational models will allow us to answer some relevant questions without the use of animals.

Why were they not suitable?

This project specifically concerns at an aspect of brain circuitry, and as a consequence there are no current viable alternatives to the use of animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The research group has extensive experience of working with functional magnetic resonance imaging and the likely requirements needed to achieve an appropriate number of viable scans. Likewise, both sets of lesions have been developed in collaboration with our partners on the grant and extensively road-tested. As a consequence, we have a very good prior knowledge of the likely number of animals required to implement the methods in our laboratory.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The study was planned with the use of the NC3R's Experimental Design Assistant (EDA). The EDA is an online tool from the NC3Rs, designed to guide researchers through the design of their experiments. It gives the researcher the ability to build a stepwise visual representation of the experiment, and thereby optimise the design with regards to animal numbers. Sources of variability in the data obtained will be further controlled by using animals of similar age sourced (as far as possible) from a single supplier.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will pilot our lesion work to ensure that the parameters we're using for the lesions are ideal for achieving our aims.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rats - methods: Functional magnetic resonance imaging of the brain, in intact animals and in animals with lesions of one of the main dopamine systems in the brain (to mimic Parkinson's disease), and in some cases an additional lesion of a part of the brain that is known to be problematic in Parkinson's disease, namely the subthalamic nucleus. The lesions will be made on one side of the brain only which will substantially limit the negative consequences of the lesions. Recovery from the lesions is expected to be unremarkable and any lasting effects are likely to be mild. The lesions themselves and the imaging will be conducted under general anaesthesia, and hence the animals will not be consciously aware of the procedures being conducted.

Why can’t you use animals that are less sentient?

The rodent basal ganglia and related structures have been widely studied and an extensive literature exists on all aspects of their structure and function. This and the fact that the circuitry is highly conserved in the vertebrate brain means our results will generalise across many species, including humans. The project is concerned with the inter-relationship between a set of brain structures found in the mammalian brain and hence the use of less sentient species (where homologous structures are not found) is not viable. As to the project itself, although the lesion aspects of the project is conducted under recovery anaesthesia, the imaging part of the project is conducted under (terminal) anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Lesions will be conducted in animals under general anaesthetic. Prior to the surgery, local anaesthetic will be injected subcutaneously into the scalp, and local anaesthetic will be

administered to the area of the wound at the end of the surgery. Immediately following surgery, systemic analgesics will be administered for pain management and animals will be placed in a heated recovery cage until fully awake. Animals are expected to make a rapid and unremarkable recovery from surgical procedures.
Animals will be observed regularly after surgery, throughout the period before scanning takes place.
For the scanning, again the animals will be anaesthetised. Physiological signals such as heart rate, blood pressure and body temperature will be monitored throughout the experimental procedures for any indication of lightness of anaesthesia. Observations for movement will be made throughout the procedures, and indices like blood pressure changes in reponse to stimuli in the experimental work, as well as common reflex tests (e.g. hindpaw withdrawal) will be used to ensure depth of anaesthesia is suitably maintained. Supplemental doses of anaesthetic will be administered as necessary.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will use the PREPARE guidelines for planning the research (Smith et al., 2018) and the ARRIVE guidelines (https://arriveguidelines.org/) for reporting the results. In addition, guidelines on the conduct of aseptic surgery (e.g. from the NC3Rs, https://researchanimaltraining.com/article-categories/aseptic- technique/) will be followed.

Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T. PREPARE: guidelines for planning animal research and testing. Lab Anim. 2018a.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As ever, I will periodically look at the NC3Rs website (https://www.nc3rs.org.uk/), and note any relevant advances and/or NC3Rs events that are relevant and upcoming. Viable and relevant advances will be trialled in the ongoing studies.

174. Mechanisms of Gene Regulation in Development and Disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Gene regulation, Epigenetics, Developmental disorders, Disease

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Genes are inherited from parents and contained within the cells of the body and contain information for making specific substances that the organism needs. We aim to understand how the activity of genes is regulated (turned up or down),how failure of this regulation leads to disease, and whether reinstating regulation can reverse or lessen the effects of disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Understanding how genes are regulated is important because incorrect regulation of genes leads to human diseases such as cancer.

What outputs do you think you will see at the end of this project?

We will generate new information in the form of data that will be published in scientific journals and presented at scientific conferences. This will provide better understanding of gene regulation in normal development and in diseases such as cancer.

Mechanisms of gene regulation that are disrupted in cancer can also cause a genetic disease called Cornelia de Lange Syndrome, which shows developmental abnormalities. Therefore, at the end of our project, we will generate a better understanding of this disease, and may potentially find new approaches to treatment.

Who or what will benefit from these outputs, and how?

In the short term (0-5 years), our work will benefit the scientific community and the general population by providing better understanding of the processes that underlie normal and abnormal gene regulation, and their relationship to health and diseases such as cancer and Cornelia de Lange Syndrome.

In the medium term (5-10 years), our work will benefit scientific communities and clinicians. It will do this by identifying approaches such as drugs or gene manipulation techniques for treating Cornelia de Lange Syndrome, and by defining the degree to which this disease can be reversed by these approaches.

In the long term (10 years plus), we will translate our basic research into clinical studies. This will benefit patients who suffer Cornelia de Lange Syndrome and related disorders.

How will you look to maximise the outputs of this work?

We will maximise the outputs of our work by actively communicating with scientists and clinicians. We will collaborate closely with internal and external research groups, and engage the public.

Scientific communication: We will present and discuss data from this work at national and international scientific meetings. We will try to get all of our data (negative, positive or inconclusive) published on scientific journals in a timely manner, so other researchers can learn from our experience. We will also use social media to alert scientific communities of our work progress.

Collaboration: We will continue our longstanding collaborations within our department, the UK, Europe, and worldwide. These collaborations will help us to meet our scientific aims, obtain clinical samples, for this project and for future work.

Public engagement: We will engage with the press and our public engagement officers to communicate our work to the public. We will directly talk to the public at events organised by our establishment or the UK scientific community. We will present our work at student open days to inspire the next generation of clinicians and scientists.

Species and numbers of animals expected to be used

- Mice: 29750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our studies will use both normal (wild-type) and genetically modified mice.

Mice provide excellent models for human diseases, and are the lowest mammalian species available for such studies. In addition, mice are the easiest mammalian species for genetic manipulation. The techniques required for generating genetically modified mice are well established, and provide a robust and reliable approach for developing new mouse lines for studying human disease (e.g. Cornelia de Lange Syndrome).

In order to understand normal development and how development is disrupted by disease, we study all life stages, including embryos, newborns, juveniles, and adults. In a limited number of studies, we will image pregnant mice, to determine effects of diet or environment exposures on their offspring.
Therefore, pregnant adults, embryos and newborns are important life stages for this project.

Typically, what will be done to an animal used in your project?

Wild type and genetically modified mice will be bred in our project and may be marked and typed for genetic modifications by ear notching.

Mice may be given substances, mainly clinically approved drugs, through their food or drink, or by injection. Mice may be immunised, which is a standard procedure to assess the functionality of immune cells in vivo.

Some mice will have their diet changed for a short period (3 weeks) so the effect of diet on development can be studied. After these treatments with substances or altered diet, some mice will be killed humanely and tissues of interest will be collected for laboratory studies. Other mice will be imaged by using equipment similar to that used for patient imaging (optical imaging and ultrasound), but specially designed for small animals. Depending on the imaging technique, a contrast agent may be injected prior to imaging, again similar to clinical procedures in humans. Other mice (e.g mice with Cornelia de Lange syndrome) will perform behavioural tasks so the function of their nervous system can be evaluated before and after the treatment. All mice will be humanely killed after completion of imaging or behavioural experiments.

We will also use pregnant mice in our work. Pregnant mice may receive treatment with substances or special diet and may be imaged. After imaging, the mother will undergo one of two different experimental pathways. They will either be humanely killed and no pups will be born. In this case the experiment will end and tissues of interest will be collected for laboratory studies. Alternatively, the mother will give birth and be humanely killed after the pups have been weaned, approximately three weeks after giving birth. No significant clinical signs are expected during this period. Humane endpoints are clearly defined - if they are suffering, the mothers will be humanely killed.

The pups will be imaged at different life stages and may be bred to examine subsequent generations. All mice will be humanely killed when the imaging is completed.
What are the expected impacts and/or adverse effects for the animals during your project?

As explained above, mice in this project will have various treatments and procedures, however, most of the procedures do not cause significant adverse effects.

1) Tissue sampling and injections may cause transient discomfort or pain.

2) Infections at sites of tissue sampling or injection are theoretically possible, but extremely rare.

3) Changes in diet may affect the weight of mice, but these effects will be minor because the changes in diet are only short term.

4) Substances given to mice are mostly clinically approved drugs, and the doses are determined to be safe by the guidance. Nevertheless, some of these substances can have side effects such as decreased appetite, vomiting, or diarrhoea.

5) Mice that are models of Cornelia de Lange Syndrome may suffer adverse effects that resemble the human disease and that affect body size, the brain, and the eyes. These adverse effects are needed for evaluating the potential treatment identified by this project. These mice will be monitored daily by animal care personnel together with veterinarians in our establishment and researchers in our group. This will ensure that any adverse effects are seen early and remain within defined limits by giving suitable treatment or by humanely killing the mice if the defined end point has been reached.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mild 80%
- Moderate 20%
- Severe 0%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals to achieve the aim of our project for three reasons. First, mice provide cells and tissues to study the mechanisms that underlie normal development and

disease. Second, mice allow us to study the interaction between genes and the environment (such as diet) by studies that cannot be performed in humans. Third, mouse models of human diseases such as Cornelia de Lange Syndrome allow us to understand human disease by experiments that can not be performed on humans.

Therefore, mice are irreplaceable experimental models for us to better understand the diseases and to evaluate the potential treatment prior to clinical studies.

Which non-animal alternatives did you consider for use in this project?

We have developed a wide range of non-animal models to address our research questions. They are cells (some from healthy humans and patients) and mini-organs (organoids) that are grown in the laboratory. We also use computer simulations and analysis of online data as non-animal alternatives.

Why were they not suitable?

Non-animal approaches are extremely valuable for our work. However, they have limitations, and are too simple to answer all questions that need to be addressed in this project.

First, we study gene expression and its regulation in development, and these models can not reflect the process of development and therefore not suitable for this aspect of our work.

Second, the symptoms of Cornelia de Lange Syndrome include learning difficulties and specific behaviours that cannot be replicated by non-animal models. Mouse models are therefore required to evaluate the efficiency of potential treatments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We estimate the number of mice required based on our experience from previous studies using similar protocols and the new questions we would like to answer in this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We draw on our significant prior experience, published work, and the NC3Rs experimental design assistant to calculate the appropriate sample size for each experiment. This will ensure that experiments use the fewest number of animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continue to develop suitable non-animal models to address some parts of our project and hopefully this will reduce the total number of animals used in this project.

In addition, we exercise tight control over our breeding colony to avoid the unnecessary production of mice beyond the numbers required to address our scientific questions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice both wild type and genetically modified. The methods for getting these mice are either by breeding (natural mating) or purchasing from a commercial supplier. Natural mating is the most refined breeding method and causes no harm, suffering, or pain to mice.

One genetically modified mouse strain bred by us is a disease model for human Cornelia de Lange Syndrome. Mice will be born with the disease and will suffer moderate symptoms as described above. Breeding these mice is the only method we can use to study this human disease.

We will use various methods to administrate substances to mice. The methods include adding substances to food or drink and injections. Food and drink routes will not cause any harm, pain, suffering, or distress. Most injections will be quickly done while animals are awake but restrained. Mice may feel transient pain but no lasting harm, similar to the reaction of humans to injections.

The imaging methods used in this project are non-invasive and therefore cause minimal harm or suffering to mice. To minimise stress, imaging will be performed while mice are asleep under general anaesthesia. Mice will breathe in a vaporised sleeping medicine and wake up quickly once the medicine is stopped. This is the most refined anaesthesia method. It allows us to control the depth of anaesthesia easily (by twitching a dial on the equipment) to provide mice a safe sleeping state. Mice also recover quickly without lasting harm from this anaesthesia method.

Why can’t you use animals that are less sentient?

In order to study human disease we need to study free-living animals with a similar level of complexity as humans, and with similar mechanisms of gene regulation. Thus, we cannot use species that are less sentient than mammals.

In some cases, we need to undertake studies on the same animals over time to observe developmental issues and evaluate treatment, and these studies cannot be done in terminally anaesthetised mice which are considered less sentient.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will take the below measures in order to refine the procedures to minimise the harms for the animals. Different measures will be applied depending on the animal experience.

1. We will closely monitor mice which have human diseases so we can spot early symptoms and act in a timely manner.

2. Anaesthesia, where used, will be of depth sufficient to prevent the animal being aware of pain arising from the procedure (e.g imaging). Mice will be kept warm and monitored regularly until they have recovered from the anaesthesia.

3. We will train mice to acclimatise to the environment and equipment before we carry out our behavioural studies and collect data. Training/acclimatisation as a refinement method will reduce the stress of mice in the new environment and benefit us by providing the reliable behavioural data.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the Animals (Scientific Procedures) Act, the ARRIVE guidelines, LASA guidelines (e.g. on administration of substances, blood sampling and aseptic techniques), the relevant published literature, as well as the PREPARE guidelines, NC3Rs website and locally published guidelines from the 3Rs advisory group of our establishment.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We consult the NC3R database, our establishment’s 3Rs advisory group, our establishment’s named animal care and welfare officers (NACWOs) and veterinarians about the best practice and potential further refinement of our procedures.

We use our weekly group meetings to discuss and update 3Rs related to this project to ensure that researchers who carry out animal work in this project are conscientious in implementing 3Rs throughout the project.

Where possible we will implement advances in the published literature and we will follow the published best practice.

To facilitate implementation of the best practice, our institute has recently employed an animal facility governance specialist who will oversee the delivery of this project and the competency of researchers in implementing 3Rs in their animal work for this project.

175. Pandemic Preparedness through Vaccine Development

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

vaccines, infectious diseases, immunology

Animal types	Life stages
Mice	adult, juvenile, aged, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This work aims to develop new and improved vaccines and vaccination regimens for prevention and treatment of infectious diseases (eg Influenza, SARS-CoV2, Nipah, RFV, MERs) that can be rapidly translated to the clinic. We aim to increase our knowledge of the immune response induced by vaccination and required for protection against infection so that we can further improve our vaccine platforms and rapidly respond to new disease outbreaks.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Vaccines are one of the most cost-effective health tools available. It is estimated that over 3 million lives per year are saved through vaccination, with vaccination campaigns leading to the complete eradication and control of some infectious diseases. Despite these successes, infectious diseases continue to cause significant morbidity and mortality world-wide because there are no licensed vaccines (eg Nipah, CCHF, MERs), current vaccines require annual changes (eg Influenza) or the disease is caused by a newly identified pathogen (eg SARs COV2). While historically vaccines were developed for prevention of infectious diseases, the ability of vaccines to stimulate the immune response is leading to development of vaccines to prevent non-communicable disease (eg cancer) but also as treatment tools once disease has developed (eg chronic viral infections). By continuing to develop and optimise different vaccine platforms we are able to apply our technology to a variety of diseases and rapidly respond to new disease outbreaks.

What outputs do you think you will see at the end of this project?

Development of new and improved vaccines and vaccination regimens against influenza and emerging pathogens (eg SARS COV1, SARS COV2, Nipah, MERs, CCHF) that can be rapidly translated to the clinic.

Increased understanding of the immune response induced by different vaccine platforms.

Increased understanding of the immune response required for protection against different infectious organisms.

Who or what will benefit from these outputs, and how?

Short-term impact of this work will be an increase in knowledge and understanding of the immune response following vaccination for the scientific community. Insight into how the immune response is induced and type of immune response required for protection against specific infectious disease will also lead to improvements and alternative approaches to vaccine design.

Medium-term impact of this work will include translation of new/optimised vaccines or vaccination regimens to small scale clinical trials, with long term impact (15 years later or more, typically) includes approval and licensing of a vaccine.

How will you look to maximise the outputs of this work?

Scientific knowledge and results will be disseminated through presentations at scientific meetings (local, national and international) and publication of results in peer-reviewed journals. In addition, we actively participate in local 3Rs meeting and local ethical review committees, sharing and gaining insight into protocol refinement methods. We have strong collaborations with vaccine (human and veterinary) researchers in the UK and international collaborations enabling the sharing of results, resources and techniques.

Species and numbers of animals expected to be used

- Mice: 15000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

We have chosen mice for these studies as they are the lowest sentient animal, with a complete immune system that closely mimics the human immune system. Performing these studies in mice ensures selection of the most immunogenic and protective vaccines/vaccination regimens that can then be progressed to GMP manufacturing and human clinical trials.

We use juvenile and adult mice as they have a fully developed immune system.

Typically, what will be done to an animal used in your project?

The majority of mice will be immunised with a vaccine preparation on up to 2 occasions and killed at a defined timepoint post-vaccination for in depth ex vivo characterisation of the immune response. For some infectious diseases, there are established models which mimic human disease, such as bone- marrow transplant through intravenous injection, therefore we can test the efficacy of our vaccine. In these instances, mice will be vaccinated with a vaccine preparation and infected with the relevant pathogen (eg Influenza virus) at set-timepoints post vaccination and monitored for development/clearance of disease.

What are the expected impacts and/or adverse effects for the animals during your project?

Most animals experience only mild adverse effects following vaccination, in line with the experience of humans (eg mild-flu like symptoms for up to 24 hours after vaccination). In studies testing the efficacy of vaccines against respiratory pathogens (eg Influenza and SARs), the predominant indicator of disease is weight loss, which peaks around 5 days after challenge before mice start to recover.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mild - 60%
- Moderate - 40%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The immune response to vaccination involves multiple, complex systems interacting in a physiological environment often involving the antibodies, T cells and cells of the innate system and therefore at present cannot be fully replicated in tissue culture. As our work aims to identify vaccines/vaccination regimens for use in humans, vaccines need to be tested in a system which closely mimics the human body. In addition, mice are the most immunologically characterised species of animal, they have proved to be excellent indicators of immunogenicity enabling the clear assessment of novel vaccines and vaccination regimens. Therefore, mice have become the standard species used for testing immunogenicity of vaccines, prior to progression into higher order species if required.

Which non-animal alternatives did you consider for use in this project?

Where appropriate we use in vitro neutralisation assays (typically lenti-viral assays) as a first step efficacy assessment. This can be used as a surrogate of protection for some diseases (Ebola,SARS- CoV-2), typically when there is not an appropriate animal model to robustly test efficacy.

We would also like to explore organoid cultures in our development of an in vitro T cell killing assays.

Why were they not suitable?

These in vitro models can only replicate or test one component of the immune system and currently the models are not in place (or sufficient) to fully replicate the immune system, although we continue to explore potential alternatives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals is based on:

the number of projects we currently have running

the number of projects for which we are planning to apply for funding

our experience of how many animals are used within each of our past projects

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We follow the ARRIVE guidelines, as well as the NC3Rs experimental design assistant, and use statistical power calculations to inform and adjust our experimental design.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Combining studies: When new vaccines require comparison to standard vaccines, we maximise the number of groups in an experiment, but to the limit of having a manageable experiment where scientific integrity will not be compromised.

Sequential sampling: we are able to monitor the immune response in the same animal over time by either sampling small amounts of blood, or through non-invasive imaging studies. This reduces the total number of animals required for a single study.

Collaborations: Challenge experiments are performed through collaborations with groups that have established challenge models (eg Ebola, Lassa, MERs), therefore we don't need to use additional animals to optimise the challenge models.

Sharing tissue: we collect multiple organs from single experiments and share samples between groups/collaborators when possible.

Efficient Breeding: Breeding will be optimised, wherever possible, to produce only the genotype required e.g. Homozygous breeding pairs, and breeding colonies monitored closely to avoid inefficient and non-productive crosses. Samples for genotyping will be taken in timely manner so that the incorrect genotypes are not kept within the colony.

Pilot studies: when establishing new models, we perform small scale pilot studies to provide important information on reactogenicity of the vaccine, or reproducibility of the assays. This data is then used in power calculations to determine optimal groups sizes required in future experiments.

Both sex mice: some differences in treatment efficacy and immunological responses have been found between genders. Following NC3Rs recommendations as well as funders, regulators and publishers we will include both mice sexes to optimize our vaccine immunogenicity and efficacy studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have chosen mice as they are one of the most immunologically well characterised animal species and therefore the standard species for immunogenicity testing of vaccines.

We predominately use non-genetically altered strains of mice, in which the immune response is well defined. When testing the vaccine efficacy, we use strains of mice and viral strains in which the disease course is well defined, therefore the scientific readout can be reached are earlier timepoints in the disease course.

We use genetically altered mice (GAA) to study the role of specific immune cell-types and genes in the immune response. Alternatively, we use mice expressing a human protein

that enabling mimicking aspect of human immune response or is required for infection of mice with human pathogens. We will only use established GAAs of known phenotypes to ensure appropriate care and monitoring protocols are in place.

Why can't you use animals that are less sentient?

Mice are the least sentient animal with a well-defined immune system that closely mimics the human body, enabling us to test the immunogenicity of our vaccines.

There are extensive sets of reagents available for analysing immune responses in mice and they have proved to be excellent indicators of immunogenicity, enabling the clear assessment of novel vaccines and vaccination regimens for improvements. With the numerous different immunological tools available, mice can be used for detailed characterisation which is not possible in higher organisms.

Using genetically modified mice with defined gene modifications provides the unique opportunity to study the role of particular genes in the induction of an immune response and required for protection.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will constantly look for more refined alternative approaches. As the primary aim is clinical translation, we will use adjuvants (or bioequivalent) that have been approved for use in humans. We use the most appropriate route of administration for each vaccine formulation, with routes and maximum volumes following published guidelines.

Our extensive experience with these vaccination studies enables the selection of most informative timepoints.

We use short term anaesthesia when the route of injection can be painful, eg intramuscular injection.

Our typical route of vaccine administration is in-line with standard administration of vaccines in humans (eg intramuscular, intradermal, sub-cutaneous) and only use alternative routes of vaccine administration when targeting the response to specific sites.

Where possible we use less virulent strains of the pathogen and/or modify the challenge dose or route of administration to reduce disease severity.

When studying the role of specific cells requires removal of these cells, generation of chimeric mice (mice that have received a bone marrow transplant) is only used if there is no alternative method that can achieve this scientific aim (eg depletion of cells with drugs or antibodies or the use of GAA mice).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The Handbook of Laboratory Animal Management and Welfare, Sarah Wolfensohn and Maggie Lloyd, Willey-Blackwell publishing, Fourth Edition (2013) in addition to following NC3Rs, LASA and ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Members of our team are active members of local ethical review committees, therefore regularly exposed to improvements and alternative approaches by researchers using similar models. We receive regulars NC3Rs and University 3Rs newsletters, we attend and participate in local 3Rs meetings. We keep up to date with the literature in both the vaccine and infectious disease field, ensuring we are aware of new or improved models that could be implemented in our research.

176. Genomics and Genome Editing of Haematological Cell Types

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Stem cells, Genomics, Genome editing, Immunology, Haematology

Animal types	Life stages
Mice	embryo, juvenile, neonate, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to develop methods of treating human disease using new technologies that allow the code of the genome to be edited precisely. We are mainly focussing on the cells in the blood including the immune system and bone marrow stem cells. We are developing strategies in a number of disease indications including correction of the mutations such as those causing forms of anaemia (e.g. thalassaemia and sickle cell disease); modulation of the immune system for treating autoimmune disease and primary immunodeficiencies; and a method of reducing the risks of stem cell transplantation through engineering the transplanted stem cells. The laboratory also has a major interest in understanding the inherited genetic risk factors that cause disease in the haematological system.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This work has the potential to cure many important inherited diseases such as sickle cell disease and thalassaemia. These cause a huge burden of disease worldwide (there are around 200,000 births per year) and many of these children die before reaching adulthood. In addition, we are developing the technology that allows us to manipulate the genome of the immune cells to treat immunodeficiencies and autoimmune disease. This has the potential to have a massive impact (5% of the population are estimated to develop autoimmune disease). Finally we are interested in developing methods to improve the safety of using genome editing in the context of cellular therapy, which is key to bringing this technology into the clinic.

What outputs do you think you will see at the end of this project?

The outputs will include:

1. Development of a new strategy for treating autoimmune disease using genome editing

2. Characterisation of the way in which genome editing occurs in haemopoietic stem cells

3. Characterisation of the way in which the mutations in the non-coding genome lead to haematological malignancy

4. Publications

5. Ultimately we aim to undertake clinical trials of the technology providing the safety and efficacy of the treatment is supported by the animal data.

Who or what will benefit from these outputs, and how?

Beneficiaries of the research include:

1. Researchers in the field of genome editing and in the specific disease areas we are focussing on will benefit from the technical advances that we make from the experimental work. We will publish the work and this will generally inform the field.

2. We hope that our work will ultimately benefit patients with inherited disorders affecting blood production and the immune system as well as patients with autoimmune diseases.

One of the approaches we have previously developed for treating thalassaemia / sickle cell disease is due to enter into clinical trials in the next one to two years. We expect that other methods will potentially enter clinical trials within the next decade if the approaches are proven to be successful.

How will you look to maximise the outputs of this work?

The outputs of the work will be maximised as follows:

1. Collaboration with other academics but also with industry where necessary. We have previously licensed technology to a large biotech company in order to gain rapid entry into clinical trials.

2. We will publish our results including unsuccessful approaches to maximise the dissemination of new knowledge.

3. Where appropriate the intellectual property from our work will be protected. This will maximise the benefit of the research because it allows external investment to rapidly translate the basic science into research.

Species and numbers of animals expected to be used

- Mice: We estimate that the proposed work will use up to 6500 mice over the next 5 years.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are planning to use mouse models because these are the current gold standard in the field for assaying haematopoietic stem cell function. The murine haemopoietic system has been better characterised than any other model organisms and it is therefore the optimal system in which to undertake this work. In order for this work to be translated into the clinic a mammalian model organism is required and the mouse is one of the least sentient mammalian species in which it would be appropriate to undertake the work.

To avoid confounding effects of puberty, we use 8-12 week old mice as "young adults", which makes our data comparable with historical datasets from other laboratories.

Typically, what will be done to an animal used in your project?

The work will involve the following procedures:

1. Drug treatments (oral / subcutaneous / intravenous / Intraperitoneal)

2. Injections (subcutaneous or intravenous) and blood sampling

3. Anaesthesia

4. Breeding and maintenance of some strains of genetically modified mice

5. Irradiation and or transplantation of human and murine haemopoietic stem cells
Surgical procedures will not be performed.
Typically, animals undergoing bone marrow transplantation and xenograft studies are likely to be on study for 16-20 weeks but occasionally they may be on study for up to 12 months.

Experiments to produce enhanced numbers of erythroid cells are short term experiments lasting typically 7-10 days.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected impacts for the animals during this project are as follows:

1. Discomfort from oral gavage but this will be transient

2. Transient pain from ear notching, injections and blood taking but the duration of the pain will be minimised to only a few minutes. Blood sampling will not exceed 10% of the total blood at any one time and will not exceed 15% of the blood volume in any 28-day period.

3. Irradiation in transplantation assays can result in radiation damage, which can cause weight loss and infection. This is generally transient, occurring around 7-10 days post treatment. Generally the animals recover rapidly and only on rare occasions will they need to be humanely killed if they exceed the permitted limits for weight loss.

4. Development of a leukaemia phenotype. When assessing off target effects of genome editing and testing variants associated with development of haematological malignancy some mice may rarely develop overt haemtological malignancy. In the vast majority of mice developing such a phenotype, the main manifestation relates to development of anaemia. Specific phenotypes may require a particular assessment method. For example, in the case of certain leukaemias (e.g. those driven by activation mutation of the growth factor receptor FLT3), mice may develop a swollen abdomen due to splenomegaly. If splenomegaly is detected, blood sampling will be carried out and if leukaemia is confirmed, mice will be humanely killed under Schedule 1 prior to onset of more overt clinical signs.

Some leukaemia’s are associated with thoracic tumours, in which case, mice may experience laboured breathing. Mice will be humanely killed immediately on identification of laboured breathing.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- 40% Subthreshold
-
- 5% Mild
-
- 50% Moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

The work aims to improve haemopoietic stem cell transplantation in humans and although in vitro models will be used we will also need to use animal models in order to answer key questions, which are critical to the success of the project. Animal models are required for the study of haemopoietic stem cells because the haematopoiesis is dependent on a complex cellular niche within the bone marrow, which it is not possible to recapitulate in vitro. Unfortunately the functional activity of blood producing (haematopoietic) stem cells from the bone marrow can only be studied in vivo. In addition, transplantation assays of human cells into genetically modified mice are the best available method of demonstrating that human haemopoietic stem cells function properly. Finally it is not possible to generate haemopoietic stem cells that behave normally from other cell types.

In the functional genomics aspects of the proposed work a small number of mice will be used because this will allow genetically modified animal models to be studied, which can provide insights, which are not achievable by other methods.

Which non-animal alternatives did you consider for use in this project?

We will extensively use cell lines and primary human cells in these projects and the use of animal's will be minimised as far as possible. In addition we have clinical expertise in stem cell transplantation and we will also use samples from patients who are undergoing stem cell transplantation where possible.

Why were they not suitable?

In vitro models are not suitable for all experiments for the following reasons:

1. It is not possible to model the stem cell niche and the complex interactions that occur inside the bone marrow in animal models.

2. Transplantation assays of human cells into genetically modified mice is the best way of formally demonstrating that haemopoietic stem cells function properly. It is essential to have documented genome editing of fully functioning haemopoietic stem cells in animal models before going on to perform human transplants.

3. It is not possible to generate haemopoietic stem cells in vitro.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Genomics experiments will be performed at least in triplicate (often with 6 replicates), which will allow differences between sample means to be detected that are greater than

twice the standard deviation. However, when it is important to detect smaller effect sizes statistical methods will be used to determine the number of replicates that are required and we will undertake pilot experiments to determine the number of animals that will be required to generate meaningful data.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have designed the experiments so that in vitro culture of human haemopoietic stem cells are used instead of mouse models. All optimisation of editing experiments will be performed using in vitro approaches and we will take advantage of our access to large numbers of stored human samples and access to cutting edge technology that allows human haemopoietic stem cells to be grown in vitro.

The NC3R's Experimental Design Assistant was used during the experimental design of this project.

We will comply with the ARRIVE guidelines (www.nc3rs.org.uk/arrive-guidelines) when publishing the work coming from this project.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The following steps will be used to reduce the number of animals being used in the project:

1. The appropriate numbers of animals in each experiment with careful experimental planning and statistical considerations to maximise the amount of information obtained from each animal e.g. serial blood sampling.

2. We plan to maximise yields of blood cells from each mouse for experimental use. For example, we will use a mouse model, which uses a drug called phenylhydrazine to expand the number of red blood cells in the spleen of the animals. This allows approximately 400 million erythroid cells to be harvested from each mouse (around hundred times more than is normally present). These can be stored in multiple aliquots and this greatly reduces the number of animals used.

3. Cryopreservation will be used to maintain smaller colonies by collaborating with the embryo and sperm freezing service team in the institute.

4. We will ensure that genome editing experiments are optimised first in cell lines and / or primary human cells. This will greatly improve the success rate of experiments and reduce the number of animals involved.

5. State of the art genome editing techniques will be used to reduce the numbers of animals required.

6. Good colony management will ensure that mice do not breed for longer than required and that colony size does not exceed that needed experimentally

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Inbred wild-type and genetically altered mice will be used in this project. Decades of scientific research and development has established the mouse as the most suitable model for studying haematopoiesis and key procedures such as bone marrow transplantation and genetic modification have been most refined in mouse as compared to other species.

Breeding and maintenance of genetically altered mice including those with expected normal phenotypes and those with immunodeficiencies. We will use standard breeding techniques to maintain genetically altered mice for investigating the genetic regulation of haematopoiesis. Conditional mutagenesis will be used wherever possible to restrict phenotypes to the relevant cell types and tissues, ensuring that any suffering is minimised.

Bone marrow transplantation assays: the most refined methods available will be used, including use of split dose radiation, which has been shown to reduce radiation complications but still obtain the level of

immune system we require for our transplant studies. and use of individually ventilated cages. Additionally, where feasible for the experimental goals, we will perform transplantation assays without the use of irradiation (the most toxic step of the procedure), e.g., by using antibody-mediated conditioning or performing transplantation in the non-conditioned setting.

Why can’t you use animals that are less sentient?

The mouse is by far the best characterised model for the haemopoietic system and it is the least sentient of the available models which have been characterised in detail. Mice are one of the least sentient mammalian species and it would not be possible to use non-mammalian models since we are hoping to rapidly translate this work for treating human patients.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To refine the procedures to minimise the welfare costs for the animals in the breeding and maintenance of genetically altered animals, we will:

1. Closely monitor genetically altered mice for signs of disease presentation or pain. Mice will be killed where pain cannot be managed.

2. Where possible, minimise breeding to reduce animal wastage.

To refine the procedures to minimise the welfare costs for the animals in bone marrow transplantation assays, we will:

1. Use split dose irradiation and closely monitor recipient mice following radiation for signs of pain and distress. Mice will be killed where pain cannot be managed.
2. Where possible, avoid use of radiation. This may be through the development and use of antibody-mediated conditioning. In certain cases, we will perform non-conditioned transplantation assays.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow published best practice guidance available here: www.nc3rs.org.uk https://norecopa.no https://www.lasa.co.uk
We will also regularly consult with our Named Information Officer, Named Veterinary Surgeon, and Named Animal Care and Welfare Officer to ensure our best practice guidance remains up to date.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will maintain our knowledge of advances in the 3Rs through:

1. Attending internal 3R meetings

2. Regularly read the latest literature

3. Regularly check up on the NC3Rs website

4. Regularly interact with the NC3R's regional manager and our Named Information Officer

As new approaches are developed which improve the 3Rs we will update our protocols and update our practice. Initially we will use pilot studies to test new techniques and these will only be adopted when they have been successfully shown to improve the 3Rs.

177. The Biology of Inhibitory Immune Receptors

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Inhibitory immune receptor, Neutrophil, Immunity, Bacterial Infection

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to understand the functions of inhibitory immune receptors in the regulation of antibacterial immune responses, and to investigate the role of inhibitory immune receptors and bacteria interactions on infection outcomes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The underlying molecular processes that regulate our innate immune responses are not fully understood and therapies to rebalance our immune responses in infectious and inflammatory diseases are required. Inhibitory immune receptors are molecules expressed in immune cells that downregulate
- or switch off - the activity of those immune cells. There is evidence that inhibitory immune receptors are important for regulating the immune response to infection and inflammation. In addition, there is evidence that bacterial pathogens have evolved mechanisms to exploit these inhibitory receptors to suppress the immune response and cause infections. Our work will permit us to understand the biological properties of inhibitory receptors in health and antibacterial defense. In addition, we shall understand how bacterial interaction with inhibitory receptors affects infection outcomes. This fundamental knowledge will contribute

to the longer-term development of therapeutics that aim to modify the immune response and/or combat bacterial infections.

What outputs do you think you will see at the end of this project?

The aim of this project is to improve our understanding of the biological role of inhibitory receptors in the biology of white blood cells called neutrophils and in bacterial infection biology. With this knowledge, we hope in the long term that this will contribute to identifying new ways to modulate the human immune response in disease situations. The data generated in this project will be published in scientific journals and presented at scientific conferences at the earliest opportunity.

Who or what will benefit from these outputs, and how?

Advancement of biological knowledge - short term

The data generated will increase our fundamental knowledge of how our immune system functions and how pathogenic bacteria cause diseases. In the short term, this will be of high value for researchers in the fields of immunology, bacteriology, and disease biology. In the mid-term, this will be of value to pre- clinical scientists. The presentation of our findings at local, national, and international conferences and in publications will ensure that our data is accessible and impactful.

Treatment of inflammation - long term

Hyper-active responses can cause tissue damage and severe inflammation. Effective strategies to reprogram hyperactive immune responses are clearly needed for many inflammatory diseases. A key step toward targeting the inhibitory receptors in new therapeutic strategies is to have robust information on the biological properties of each receptor. However, this fundamental knowledge is lacking. The data generated here will contribute to the identification of the most appropriate inhibitory receptors to target in immunotherapeutics that will benefit patient health. This has the capacity to benefit pre-clinical scientists, industry, and public health in the long term.

Treatment of bacterial infections - long term

Bacterial infections are a leading cause of human mortality. Thus, new effective strategies to combat bacterial diseases are urgently required. Re-harnessing the power of our immune system is a promising therapeutic strategy to rebalance immune responses in disease situations. Alternatively, targeting bacterial pathogenicity mechanisms is an attractive option to combat bacterial infections. The data generated here could contribute to the development of new therapeutic strategies for the treatment of bacterial infections. This has the capacity to benefit pre-clinical scientists, industry, and public health in the long term.

How will you look to maximise the outputs of this work?

We aim to maximize the output of our work through collaboration, scientific communication, and public engagement.

Collaboration

We closely collaborate with other scientists in the inhibitory receptor biology field and bacteriology fields. Our close collaboration with academic collaborators will ensure the

work is performed using the most robust and effective approaches available. We will frequently discuss our work with collaborators to maximize the dissemination of our knowledge.

Scientific communication

Our findings will be communicated to other scientists at scientific conferences and through publication. In addition, we will communicate our results in the context of the broader field through the publication of review articles. We will publish our findings as they are, to ensure the same experiments are not repeated in other laboratories without justification.

Public engagement

We will engage in public engagement events within our institution so that members of the public can learn about research. Press releases will be coordinated and aligned with the publication of our research findings. Through these events, we will keep the public informed of our research.

Species and numbers of animals expected to be used

- Mice: 1000 wildtype mice, 1500 GA mice.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse will be used as the model species on this license. It has been selected as it represents the best animal model available for the purpose with the greatest likelihood of achieving the outlined research objectives. The mouse model is further justified on the basis that genetically altered mouse lines expressing human inhibitory immune receptors have been recently developed. This allows mechanistic studies of their functions. We will breed genetically altered animals and will use animals of all life stages.

Typically, what will be done to an animal used in your project?

Most animals will be exposed to an inflammatory molecule (by injection into the abdomen or a superficial vein) or bacteria (by injection in the abdomen, superficial vein, or by inoculation via the nose, esophagus or vagina). To understand the effect of inhibitory immune receptors, we will sometimes administer substances to the mice that interact with the receptor before the inflammation or infection. These substances may include antibodies or characterized ligands of the immune receptor because it is important to understand how the inhibitory receptor functions to regulate immune responses. These substances will be administered by injection into the abdomen or into a superficial vein for the easiest rapid spread into the bloodstream. We will collect blood samples typically 2 times, but sometimes up to 6 times to monitor the immune response. Animals are visually monitored at least once daily to ensure the inflammation or infection is not causing ill health. Animals would typically be humanely killed at early (by 8 hours after inflammation or infection) and later timepoints (between 2 and 14 days after infection), which may include under deep anesthesia so that large volumes of blood can be collected for

analysis. A range of samples will be collected to study the immune response during inflammation or infection in detail

What are the expected impacts and/or adverse effects for the animals during your project?

It is not expected that GA mice bred on this project will display short-term or long-term adverse effects. The majority of animals used in our project will be challenged with an immune stimulant to induce inflammation or challenged with bacteria to induce bacterial infection. Our experiments are designed to study the acute phases of inflammation and infection before animals progress to severe and/or prolonged systemic inflammation of infection. Nonetheless, the animals will display moderate adverse effects including weight loss, abnormalities in behavior or physical appearance (somnolence (drowsiness), hunched appearance, piloerection (raised fur), reduced activity), or alterations in feeding, discomfort/distress. This will vary depending on the immune stimulant, bacteria (species, strain), dose and administration route. We will humanely kill animals when they start to display adverse effects, to prevent further suffering. In addition, we will immunize with antigens and refined adjuvants to produce antisera that can be used in our experiments. Immunizations may induce localized inflammation for up to 2 days. In rare instances, we may need to immunize animals with antigens in combination with less refined adjuvants that can cause granulomas, ulceration, and abscesses at injection sites for up to 2 days. Animals may be treated or humanely killed depending on the extent of the symptoms. The

project licence provides a clear outline of the adverse effects and limits that will guide the decision to either treat or humanely kill an animal.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild (85%) or moderate (15%).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The immune response is formed from multiple immune cell types that are never found in isolation in vivo (in the living organisms) and from complex signalling mechanisms. Thus, circulating immune cells are diverse in their behavioural and functional properties. Therefore, investigations that are mechanistic and under physiologically relevant conditions are required to uncover the functional properties of human inhibitory immune receptors in health and disease. This requires the functional comparison of immune cells

that have been genetically manipulated to express (produce) or not express a specific inhibitory receptor.
Our primary objective is to investigate the functional properties of inhibitory immune receptors on neutrophils because they are the most abundant immune cell in circulation and provide the first line of defence against invading bacteria. However, it is not possible to genetically manipulate primary human neutrophils. In addition, there is no human cell line that recapitulates the expression profile and functions of human neutrophils. Therefore, no alternative that does not use animals would allow mechanistic assessments of the functions of inhibitory receptors on human neutrophils.

The immune response to microbes is formed from a complex interplay of host- and microbe-derived factors. This complexity cannot be thoroughly replicated in vitro (without using animals). Thus, our research into antibacterial immunity and bacterial virulence mandates the use of in vivo experiments as there is currently no in vitro system which could generate meaningful data.

Which non-animal alternatives did you consider for use in this project?

We have considered using 1) primary human immune cells, 2) human cell lines, and 3) primary mouse/rat immune cells. However, these options do not allow mechanistic assessment of the specific functions of inhibitory receptors or bacteria-inhibitory receptor interactions in immune responses.

Why were they not suitable?

Primary human neutrophils have a short lifespan and cannot be genetically manipulated. There is no good human cell model of neutrophils that recapitulates inhibitory receptor expression profiles and functions of primary human neutrophils. Therefore, primary neutrophils and cell lines are not a suitable replacement for animal models in our study. Primary mouse/rat immune cells do not express human inhibitory receptors and therefore are not a suitable model either. The results gained from any in vitro method do not reflect the complex interactions in the whole animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is a 5-year project in which we propose to breed around 3 different genetically altered mice lines. This will allow us to perform experiments to test the role of different inhibitory immune receptors in health, immunity, acute inflammation, and infection biology. We have estimated mice based on the number of genetically altered mouse lines, the anticipated numbers of experiments, the numbers of experimental groups, and the numbers of mice in each group. The estimates are based on similar studies that have been conducted in the past. The projected number of animals reflects the number of animals necessary to achieve the scientific objectives outlined in the programme of work. Our estimates of

animal numbers have also been reviewed by other scientists who work in the area to give us confidence in them.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We discuss our experimental design with collaborators and other academics that are more experienced to minimize the number of animals used. We plan our experiments to gain the most data and scientific information from each animal and to reduce the number of animals that we need to use. Our planning will include 1) analyzing data from experiments that are published, 2) analyzing experiments from other projects, 3) using tools such as the NC3Rs Experimental Design Assistant to plan and organize all parts of the experiment, 4) using power analyses and advice from biostatisticians to calculate the minimum number of animals required to detect statistical differences between groups.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will take all reasonable steps to reduce the number of animals used in our project without compromising the quality of data collected and without compromising the objectives of the project. The majority of animals used in this project will be genetically altered animals which we will carefully breed in our facilities to minimize waste and to ensure that every animal can be used in experiments. The majority of experiments will be performed with a small number of animals to ensure fewer animals may experience unexpected adverse effects. We will collect as much information from every animal as possible. For example, in some experiments, our primary objective is to quantify neutrophil counts in the blood and in the intraperitoneal cavity (the space within the abdomen that contains the intestines, the stomach, and the liver that is bound by thin membranes) after an immune challenge. In these experiments, we will collect and store serum samples and tissue samples that can be analyzed in the future or by other researchers. This will reduce the requirement to perform future experiments and therefore lead to a reduction in animal usage over time.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse will be used mouse models as the model species on this license. It has been selected as it represents the best animal model available for the purpose with the greatest likelihood of achieving the outlined research objectives. The mouse model is justified on the basis that genetically altered (GA) mouse lines expressing human inhibitory immune receptors have been recently developed. We need such GA models for our studies. Other species do not have such GA models available. These models have been validated already so this will reduce the need to develop new GA animal lines. The mouse model is

further justified on the basis that immune challenge and bacterial infection models reflect the key features of innate immune responses in humans.

The procedures of the outlined mouse models are of mild or moderate severity if allowed to proceed to the symptomatic stage of infection, though this is not the intention of most experiments planned in this study. We follow protocols that have been established and refined over several years to cause the least pain, suffering, distress, or lasting harm to the animals. The scoring systems for animal harm that have been developed for the studies contained in this license ensure the minimization of suffering, especially during the critical phase of the studies. This is of particular importance for models of systemic inflammation and bacterial infections that can be rapidly progressive. The implementation of well-established models will reduce the pain, suffering, distress, or lasting harm to animals.

Why can’t you use animals that are less sentient?

We need to work with a mammalian species that express human immune molecules that recapitulate the expression profile of the human system. Mice are selected because they are the only species for which the required GA animals have been developed and validated. We also need to work with adult animals as we need to study animals with a fully developed immune system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We follow established protocols that have been developed and refined over several years. The animals will be monitored daily by 1) central biological services staff (who monitor food supply, water supply, and clean bedding) and 2) researchers. This monitoring will highlight any signs of deviation from normal patterns of behavior. Minimization of pain, suffering, distress or lasting harm will be assured using rigorous health scoring sheets and frequent monitoring during the critical phase of the studies.
Sterile procedures are used for minor surgeries and pain relief is given where necessary.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

LASA guidelines and ARRIVE guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about advances in 3Rs by following external sources including the National Centre for Replacement Refinement and Reduction of Animals in Research resources (website https://www.nc3rs.org.uk/) and NCRI guidelines, and through reading new literature relevant to the experiments outlined in the programme of work. All PIL working under this PPL license will attend these meetings and frequent advice regarding the well-being of animals will be sought from the NVS and animal care staff. We will further refine our experiments through discussions with collaborators and in the broader academic network.

178. Characterizing and Correcting the Effects of Hearing Loss on Neural Coding in the Auditory System

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words
hearing, hearing loss, neurophysiology

Animal types	Life stages
Guinea pigs	adult
Gerbils	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to better understand the links between hearing loss and impaired auditory perception by characterizing the effects of hearing loss on the neural code -- the brain's internal representation of sound. We also aim to develop improved processing algorithms for assistive listening devices that can restore the neural code to normal after hearing loss.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Hearing loss is one of the world’s most widespread and debilitating chronic conditions. It is a top-5 contributor to the overall global burden of disability; the leading modifiable risk

factor for dementia; and costs nearly $1 trillion per year. Its full impact, however, is difficult to capture with statistics. Because it impairs speech perception, hearing loss impedes communication and leads to social isolation and associated decreases in quality of life and wellbeing. Unfortunately, current treatments provide only limited benefit for speech perception in many real-world settings. We will exploit recent advances in neuroscience to gain insight into how the different physiological changes associated with hearing loss interact to produce specific perceptual deficits, and to test new ideas for technologies that can correct these deficits.

What outputs do you think you will see at the end of this project?

This work is expected to provide new information about the neural underpinnings of impaired auditory perception. The primary expected benefit is the publication of new scientific knowledge about the specific ways in which hearing loss distorts the brain's internal representation of sound. Additional outputs and benefits are expected to include new algorithms that could be used in hearing aids or other sound-processing devices to improve perception of speech in noisy environments and/or enjoyment of music for hearing-impaired listeners.

Who or what will benefit from these outputs, and how?

In the short term, outputs from this project will benefit other scientists seeking to better understand hearing loss. The project will also benefit students and other trainees who work on the project by

providing them with an opportunity to develop the skills and knowledge required to conduct high- quality, clinically-relevant research. The knowledge gained from the project will be shared with other specialists through publication in scholarly journals and presentation at national and international conferences, and with a wider audience through public lectures and lay media.

In the medium and long term, outputs from the project will contribute to development of novel tools for scientific research and new technologies for improving human hearing. The project will also benefit the UK economy in the medium term by helping to develop a skilled workforce for scientific research.

How will you look to maximise the outputs of this work?

Outputs will be disseminated through open-access research publications, and novel research methods and software that are developed during the course of the project will be shared through open-access repositories. Impact will be maximized by working closely with clinicians and people with hearing loss to ensure that studies are designed to be relevant to human health and wellbeing.

Species and numbers of animals expected to be used

- Guinea pigs: 100
- Gerbils: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our work requires an animal model with hearing that is similar to that of humans. While all mammals share the same basic auditory architecture, different species vary widely in the range of sound frequencies to which they are sensitive. This variation has important functional implications because only certain animals can actually hear human speech. Among the possible models with the required sensitivity -- monkey, cat, chinchilla, ferret, rabbit, guinea pig, gerbil -- the latter are among the most widely used and are also the easiest and most cost effective to work with. There are some studies, however, for which the geometry of the gerbil cochlea may be inappropriate. For these studies, we will use guinea pigs instead. We will work only with adult animals, using young adults in most cases and aged animals when studying age-related hearing loss.

Typically, what will be done to an animal used in your project?

Our studies typically begin with minimally- or non-invasive procedures to assess hearing, just a human would undergo during a routine visit to an audiologist. Animals are anesthetized during these procedures if they are at all invasive and given adequate time to recover before additional procedures are performed. We then typically induce hearing loss in a subset of animals (for example, through noise overexposure), again using anesthesia when necessary and allowing appropriate time for recovery. Most of our studies then finish with a final recording session under terminal anesthesia. During these sessions, animals undergo a surgery in which we perform a craniotomy and insert silicon probe electrode arrays into the brain. We then record neural activity during the presentation of sounds for 10-12 hours before schedule 1 termination. In some cases, when we need to verify that the use of anesthesia is not confounding our results, we secure the electrode with dental cement and allow the animal to wake up after the surgery. After an appropriate recovery period, we then measure neural activity using the implanted electrode for several hours per day, sometimes under restraint, over the course of several weeks before a final terminal recording session is performed under anesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not typically observe any adverse effects after recovery from our procedures. We provide analgesics and monitor behavior in the days following any procedure. Animals with implants are inspected daily to ensure that any signs of weight loss or malaise are quickly identified and treated.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most of our animals will experience procedures of moderate severity, including induction of hearing loss and repeated minimally-invasive procedures performed under anesthesia with recovery. A small fraction of animals (~10%) will also undergo intracranial electrode implantation under anesthesia with recovery.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our experiments must be performed in animals because the aspects of hearing and hearing loss that we are studying depend on the fine details of neural activity patterns that cannot be observed in humans through non-invasive recording methods and cannot yet be replicated in computer models. We also require the full auditory system to be intact, which precludes the use of ex vivo or in vitro approaches.

Which non-animal alternatives did you consider for use in this project?

We always consider the possibility of using computer simulations or non-invasive recordings in humans instead of using animals.

Why were they not suitable?

Many aspects of the auditory brain are not understood well enough to be simulated accurately. We do, however, use computer simulations whenever possible to refine our hypotheses before using animals.

The aspects of auditory processing that we are studying are dependent on detailed patterns of neural activity at micrometer spatial and millisecond temporal scales. Neural activity cannot be monitored at these scales with non-invasive methods and, thus, recordings of brain activity in humans, e.g. via electroencephalography (EEG), are not sufficient.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These estimates are based on our recent history of animal use under our previous project license. If anything, these numbers are overestimates; methods for intracranial measurement of neural activity continue to improve in ways that allow us to obtain more data from individual animals, potentially reducing the total number of animals required.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For each of our individual studies, we use software tools (such as the NC3R's Experimental Design Assistant) and seek external assistance (such as through the Biostatistics Advice and Collaboration service) when designing our experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We use both male and female animals in our experiments, both to minimize the number of animals bred and to avoid sex bias in our results. We also use computer simulations to refine our hypotheses before running any animal experiments, and to aid in our estimation of the number of animals required for an individual study (i.e. by estimating likely effect sizes or variability). In many of our studies, data points are neurons or neural networks, not animals; therefore, although results must be confirmed in multiple individual animals, we can minimize the number of animals needed by maximizing the number

of neuronal recordings obtained per animal, which we do primarily through the use of large-scale recording technologies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Most of our studies use young adult gerbils. We sometimes use guinea pigs in cases where the specifics of gerbil anatomy render them unsuitable, and we sometimes use aged gerbils when we are studying age-related hearing loss.

The methods we use can be grouped according to their purpose: hearing loss induction, hearing assessment, and neural activity measurement. For hearing loss induction, we choose our method according to the particular form of hearing loss that we are trying to model. For most of our studies, we are able to use methods with relatively low severity, such as ear plugging or noise overexposure. More severe methods such as intra-aural procedures are only used when necessary to create specific cochlear pathologies. For hearing assessment, all of our methods are minimally or non-invasive and are comparable to what a human would experience in a clinical examination. For invasive neural recording, the vast majority of animals will undergo only a single terminal session under anesthesia. Repeated recordings in awake animals are kept to the minimum required to validate that key results are not an artifact of anesthesia.

All animals, whether or not they are part of an active study, are examined at least once per day to ensure their general welfare as well as to monitor potential adverse effects of recent procedures they may have undergone. Once an animal is moved onto an active study, they are progressed to the final terminal recording session as soon as possible (typically within a few weeks, and longer only when required to assess the long-term effects of hearing loss on auditory processing). All animals are provided with enriched group

housing, except in rare cases when single housing is required to protect an intracranial implant.

Why can’t you use animals that are less sentient?

Our research goals require an animal model of human hearing (i.e. an animal for which we are confident that our results will also apply to the humans that we are intending to model). Our choice of animal model is the least sentient possible that satisfies this requirement.

Most of our intracranial recordings are performed under terminal anesthesia. However, given that anesthesia can have profound effects on brain dynamics, it is important to also validate any novel findings in a small number of awake animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We already use and will continue to use best practices for monitoring, post-operative care and pain management. We will seek to improve these methods through consultation with our NVS and advice from NC3Rs, which is sought before beginning each new study.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow guidance from NC3Rs and LASA (Laboratory Animal Science Association) to keep up to date with knowledge of the use, care and welfare of our animals and strategies for refinement, reduction and replacement.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We check the NC3Rs website periodically and subscribe to their updates. We are also members of several different groups that meet regularly to share advances in best practice and opportunities for further training, and provide academic leadership for a departmental group overseeing welfare and other matters related to in vivo experiments.

179. An Inducible Pig Model of Microvascular Disease

Project duration

3 years 0 months

Project purpose

(a) Basic research

Key words

brain, blood pressure, blood vessels, heart, microvascular disease

Animal types	Life stages
Pigs	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To assess the potential of a genetically modified pig designed to develop high blood pressure (hypertension) in response to a hormone-like molecule called endothelin, and then to see if endothelin- induced hypertension has any effects on brain white matter." If successful this model offers future opportunities to test therapies for brain, heart and kidney diseases that result from chronic hypertension.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The effects of hypertension on small arteries ("microvessels") are a common cause of ill-health in older people. Recent statistics suggest that at least 7 out of 10 older people with dementia have some degree of brain microvascular disease, indicating the latter is a common contributing factor.

Understanding how this comes about is incomplete: the molecules involved, their relative "toxicity" and the "cause and effect" relationship of blood vessel change and hypertension are all unknown.

Therefore, there are few medications available to treat microvascular disease effectively. An accurate model of the human disease in a species with similar brain size and function would facilitate the improvement of current treatments and offer opportunities to assess new therapies. A successful porcine model of microvascular disease could increase the understanding of the molecules and mechanisms involved and provide a valuable method for testing new therapies, including drugs.

What outputs do you think you will see at the end of this project?

This project seeks to establish the value and limitations of a new pig model of hypertension. If successful, an entirely new transgenic pig model of endothelin-based cardiovascular disease will become available. The initial proposal is restricted to testing whether physiological variables (such as blood pressure) change in proportion to endothelin levels in the animals - and for up to 8 days after the gene controlling endothelin transgene production is activated. During this time tests will be conducted to identify brain microvascular lesions. It is anticipated that future studies will expand this investigation of controlled endothelin gene overexpression along with the testing of potentially useful drugs. We hope that these modified pigs will be used by ourselves and other groups in future studies of endothelin-related cardiovascular disease, including studies of drugs like zibotentan and ambrisentan which block the effects of endothelin at the ETA receptor (an effect which normally results in blood vessel constriction). Beyond academic studies of mechanisms and pathology, promising drug candidates will be developed and tested in collaborations with commercial partners.

Who or what will benefit from these outputs, and how?

The outputs from this project will benefit four groups.

First, studies of both normal function and diseases that involve endothelin-1 will benefit from the availability of a new large animal model. The current literature on large experimental species (primarily dogs and pigs) describes studies performed 30 years ago. There are few studies of the effects of endothelin on the blood vessels of larger species that have more human-like brains with extensive white matter. The proposed model will provide a platform to study dose-dependent actions of endothelin-1

Second, other diseases involving changes to small blood vessels will benefit. The current project is primarily an investigation of the impacts of vascular actions of endothelin-1 on the brain. However, the endothelin system is important in the blood vessels of other organs, notably the heart, kidneys and lungs, and so successful model development will allow future studies to explore mechanisms and test therapies relevant to these organs. For example, changes in the small blood vessels that feed the heart muscle cause angina, a common debilitating condition in older humans – which, like dementia, is not always well-managed with currently available drugs. These are disease areas in pressing need of evidence-based interventions and therapies. As altered blood flow in the brain is the foremost cause of dementia, future studies will likely benefit from this novel platform.

Third, pharmaceutical companies with a commercial interest in drug development programs will benefit. Endothelin and its receptors are of interest to several pharmaceutical companies, three of whom have already provided letters of support for this project.

Fourth, and most importantly, this project has the potential to benefit older patients affected by microvascular disease. Despite the international prevalence of diseases of small blood vessels, availability of effective treatments remain limited. Recent studies provide strong evidence for involvement of the endothelin system in human vascular disease, including hypertension and specifically in microvascular disease. Indeed, current data link activity of the gene encoding endothelin with an increased risk of at least five vascular disorders, including coronary artery disease; migraine; and hypertension. It seems highly probable that the endothelin system is an important component of cardiovascular disease in older people.

Our model will benefit microvascular disease patients i) by increasing understanding of endothelin- driven pathological processes, and ii) by providing a model for preclinical testing of drugs and other therapies.

How will you look to maximise the outputs of this work?

The outputs of this work will be reported in peer- reviewed publication and at scientific conferences. We are also initiating a blog to report progress informally. We are already engaged with collaborators at other academic institutions within the UK and USA, and have letters of support from three pharmaceutical companies) with a commercial interest in hypertensive disease.

These inducible ET1 (iET1) pigs will be used to study endothelin-driven cardiovascular disease in both academic studies of mechanisms and pathology and commercial projects focussing on drug development and testing. We have support from expert groups in dementia and heart disease. For our own purposes, iET1 pigs will be used for examining brain and heart microvascular lesions and testing interventions including existing drugs for possible repurposing, or biological agents (RNAs, nanoparticles, viral vectors).

Large animal models have the potential to provide a valuable translational bridge between current in vitro or rodent studies and early-phase clinical trials. For example, pre-clinical data in a large animal species has recently accelerated translation of biotherapeutic agents for a rare childhood dementia. A recent market review of pig and sheep models, carried out by an IP/technology management consultancy identified a global Animal Model Services market of $650 million. This report highlighted an opportunity to work with the customised animal model producers within the industry, as few companies have the expertise to create genetically altered large animal models themselves.

Species and numbers of animals expected to be used

- Pigs: 40

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using pigs for this project because their brains, whilst smaller than a human brain, are similar in many other ways to the human brain.

Typically, what will be done to an animal used in your project?

Genetically modified pigs will be created under a separate licence.
Initially we will take a blood sample and small skin biopsy (circa 5 mm diameter) from each experimental animal to establish a baseline for ET1 expression. Later, the pigs will be fed an antibiotic (doxycycline, or similar) in their feed which will induce expression of the transgene. This in turn is expected to result in increased endothelin levels and an increase in blood pressure. It is hoped that adjusting the amount of doxycycline administered may allow some control over the degree of hypertension produced. A few days (Three, up to a maximum of 8 days after the first doxycycline dose, the pigs will be anaesthetised for an MRI scan to examine the blood vessels in their brains. They will then be killed without recovery from anaesthesia. At post mortem further tissue samples will be taken and the brains removed for thorough microscopic analysis.

The inducer drug, doxycycline, is rapidly absorbed from the stomach after oral dosing. `Doxycycline- mediated induction of the transgene occurs within 24 hours. In rodents and monkeys the vasoconstrictor effects of injected ET1 on brain blood vessels occur within a few hours. Based on this it is believed that induced ET1 mRNA and subsequently peptide expression will occur within 1-2 days, with vascular effects expected a few hours thereafter. Scheduling the MRI scan for a maximum of 8 days after induction was chosen to allow for any under-estimate in this analysis, and to allow brain lesions to become evident on MRI (which may take a few days).

What are the expected impacts and/or adverse effects for the animals during your project?

High blood pressure in humans often has few associated symptoms. Indeed, many people living with high blood pressure are unaware of the problem. However, chronically elevated blood pressure is associated with diseases such as dementia, heart attack, and stroke.

There is strong evidence that the vasoconstrictive effects of ET1 are dose-dependent. It is anticipated that induction of ET1 expression in pigs will have a hypertensive effect. This will be achieved in this study by giving doxycycline in feed,

In the proposed experiments, a small cohort of experimental pigs will receive a low doxycycline dose in feed, which should induce modest levels of ET1 expression. The principle anticipated effect, which will be recorded, will be an increased blood pressure, which will be measured using oscillotonometry, i.e., with a standard blood-pressure cuff applied to the animal’s tail or leg. An MRI scan will also be taken to identify possible vascular changes in the brain, including white matter lesions and microbleeds.
Adverse welfare effects are not anticipated. If the initial dose of doxycycline is ineffective (in terms of raising changing blood pressure and other physiological variables) a second small cohort of pigs will receive a higher dose and the effects (beneficial and adverse) recorded. Conversely, if adverse effects are observed after low dose doxycycline, subsequent animals will receive an even lower dose. This stepped approach should allow end-points to be achieved without adverse effects. However, as this is a new model it remains possible that the initial degree of transgene induction may be greater than predicted, and that severe hypertension produces signs such as rapid shallow breathing, a loss of awareness, head-pressing, or in the worst cases, seizures or collapse. In the event that such unintended clinical signs are observed, the affected animals will be culled. It is not anticipated that animals will experience more than moderate clinical signs during the

induction process, throughout which the animals will be monitored closely for adverse clinical signs.

Expected severity categories and the proportion of animals in each category, per species.
What are the expected severities and the proportion of animals in each category (per animal type)?

All animals are expected to experience a moderate severity level. All animals will have a skin biopsy taken, and some animals may develop adverse clinical signs during the course of induction.
Cumulatively it is expected that animals will not exceed a moderate threshold.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

A meaningful investigation of the actions of endothelin-1 requires an intact mammalian cardiovascular system – and so, intact animals. Previous studies have used mice but this study will use pigs because the porcine brain and its blood vessels are more similar to those of the human being.

Which non-animal alternatives did you consider for use in this project?

Previous studies have used in vitro methods, e.g., cell cultures, or isolated arteries to assess the vascular actions of ET1. In this study cell culture experiments have been used to test the gene induction system allowed the effectiveness of the cDNA construct to be confirmed before injection in pig embryos.

In addition, the antibodies to be used for immunohistochemistry in the proposed study have already been characterised using ex vivo pig brain samples from previous experiments.

The use of cadaveric human brain tissue to examine the effects of endothelin on brain microvessels and allow the mapping of marker expression in brain tissue was also considered.

Why were they not suitable?

The use of cadaveric human brain tissue would not have allowed the dynamic in vivo actions of endothelin to be studied, or related to concentrations of endothelin in blood or tissue.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.
How have you estimated the numbers of animals you will use?

Initially, small cohorts (typically, 6 transgenic animals, 4 of which will be induced and 2 of which will function as controls) will be used in the doxycycline dose-ranging studies designed to identify the induction dose required to produce clinical effects, i.e. arterial hypertension, without significant adverse effects. Measurement of blood pressure will be initiated at least 2 days prior to transgene induction in order to establish an internal control for the experiment. Dose-ranging will begin with a low dietary doxycycline dose, that has previously been reported to induce transgene expression in pigs. If analysis confirms that an appropriate phenotype has been achieved without adverse events, then a fully powered experiment in adult animals will be conducted. If further optimisation is required then induction may be repeated in further small cohorts with higher or lower doxycycline doses as appropriate. If no licensed procedure is performed on control animals, e.g., only BP measurement, these may be either used in subsequent experiments or culled by schedule 1 to provide comparator tissues for analysis.

The results of initial experiments will be used to perform a larger experiment on a cohort of adult pigs (6 transgenic and up to 6 controls), which may be repeated on one further occasion. Control animals will not carry the endothelin transgene and will serve to control for the unlikely possibility of unintended effects associated with the inducing drug, as well as comparison with expected ET1 mediated damage in terms of physiology (BP, electrolytes), imaging changes on MRI, and neuropathology post mortem.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The lentiviral construct that will be used to produce the transgenic animals has already been produced and tested by transducing the pig kidney cell line PK15. Adding doxycycline to the cell culture medium results in induction of transgene expression, detected above background using qRT-PCR. These in vitro data provide confidence that a dietary doxycycline supplement will result in transgene expression in the iET1 animals.

A previous study attempted to achieve transgene (RANKL) expression in pigs using doxycycline in feed. Low doses (6.3 mg/kg per day) failed to promote transgene expression measurable above background levels. A dose of 12.5 mg/kg per day produced low detectable transgene levels in plasma, while 25 mg/kg caused a further elevation of expression. These doses will be used in the initial dose- ranging studies to refine the experiment and reduce the number of animals that may otherwise have been required

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

It is proposed that the numbers of animals proposed are the minimum required to achieve the objectives of both the dose-finding pilot study and the subsequent project.

Tissue samples from other organs (e.g. hearts, kidneys, liver and lungs) will be collected and banked for analysis before future projects, in order to optimize animal use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Transgenic pigs with a doxycline-inducible endothelin 1 transgene, and non-transgenic control pigs will be used. Blood and, or skin biopsies will be taken from each animal before induction of transgene expression to establish a baseline for each experiment. To establish baseline values, non-invasive blood pressure will be taken on each animal up to twice per day until the act of measurement is

accepted by the animal and values are within the range considered normal under pigs of similar age and size. The antibiotic doxycycline will be added to the animals feed to induce transgene expression. After a maximum of 8 days, animals will be anaesthetized to allow brain MRI scanning and the collection of further blood samples and tissue biopsies. The anaesthetized animals will then be killed by overdose of anaesthetic and when dead, their brains and other tissue samples collected for histological analysis

Why can’t you use animals that are less sentient?

Previous work has been performed in rodent models. However, the size and complexity of the rodent brain and associated vascular system are less similar to that of humans than are those of the pig. A large animal model of microvascular disease is currently an unmet need in translation of data from in vitro and rodent models to clinical application.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal husbandry, surgery and anaesthesia will take place at our universities fully-equipped centre for large animal experiments.

Pig anaesthesia and surgery will be overseen by the applicant or his designated representative

Daily welfare checks will be carried out by the NACWOs or trained PIL holders. Upon doxycycline induction and for initial experiments monitoring frequency will be increased (at least once every 8 hours).

An initial stair-cased dosing method (see section on Reduction) will be used in small cohorts of animals to refine the doxycycline dose required to achieve the desired phenotypic outcomes ahead of a fully powered study.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will refer to the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) during the planning stages and report our studies according to the ARRIVE 2010 guidelines (Animal Research: Reporting of In Vivo Experiments). The principles laid out in these resources will enable us to design and conduct the experiment in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Animal welfare will be prioritized throughout the study by incorporating all permissible refinements identified by the establishment's Named Veterinary Surgeon and other veterinary colleagues.

The NC3Rs website (https://www.nc3rs.org.uk/) will also be consulted and full advantage taken of the annual seminar day organized by the University's Biological Services to identify changes in best practice and methods to improve animal welfare.

Any adaptations will take place following discussions with the NVS and NACWOs before incorporating them into the study plan.

180. Regulation of the Stress Response in Birds

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
    - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

chicken welfare, stress response, neuroscience, endocrinology, stress resilience

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	neonate, juvenile, adult, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to better understand how the stress response is regulated in birds. We will investigate the role of different subdivisions of the forebrain (i.e. the main brain hemispheres) in regulating the stress response, as well as the influence of physiological, environmental, and/or genetic factors on the strength of the stress response.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The poultry industry provides an important proportion of our dietary protein, both in the form of chicken meat and eggs. Both laying hens (39 million in the UK alone) and broiler

chickens (more than 1 billion per year in the UK; chicken accounts for roughly 50% of all meat eaten) are farmed in high-intensity housing systems, often with flocks of many thousands of birds, or at high densities in enriched cage systems. Although these systems provide an improvement in animal welfare over the traditional battery cages, it is still unclear how well the birds cope with these modern housing systems, and therefore how their welfare is affected. It is therefore important that we can measure the welfare of the animals, so we can optimize the housing conditions. Given that welfare is (at least partially) about the subjective experience of the individual, it is also important that we rear the animals in a way that makes them as stress resilient as possible, allowing them to cope better with industry conditions.

To assess welfare and stress resilience, we need to measure how the animals' stress responses are activated by their housing and husbandry conditions, and how this response is changed by different rearing and housing conditions. We therefore also need to understand how the stress response is regulated. Many aspects of the stress response (e.g. stress hormones) are activated by a wide range of conditions, including physiological "stressors" like low blood sugar, psychological stressors like a dominance fight, and even by positive conditions, like exploration of a novel, complex environment.
Understanding the regulation of the stress response will allow us to:

(1) develop novel markers of stress and stress resilience, potentially ones that are more specific to the kind of stressor (e.g. psychological vs. physiological)

(2) understand how we can make the birds more resilient to certain stressors, either by changing environmental conditions (e.g. during rearing) or by breeding birds for stress-resilience traits.

To achieve the first of these goals, we need to understand more about how the brain regulates the stress response. Much is known about this in mammals, but not in birds. Given that the forebrains of birds and mammals are very different, the knowledge from mammals cannot be automatically transferred to birds. For the second goal, we can explore different environmental manipulations (e.g.

during early development or during adulthood) and assess their effects on the responses to acute and/or chronic stressors.

What outputs do you think you will see at the end of this project?

The forebrain is the main part of the brain which interprets the world around us and therefore shapes our responses to psychological stressors. At the end of this project, we will have a better understanding of which parts of the forebrain in birds are involved in regulating stress responses and what their roles are in this.

Additionally, different environmental manipulations may make the birds either more or less resilient to stressors. We will investigate such environmental manipulations (e.g. rearing methods, feed supplements) and their effect on responses to acute and/or chronic stressors in chickens. This will allow us to make recommendations about ways to reduce the experience of stress in poultry and thereby improve their welfare.

Who or what will benefit from these outputs, and how?

Comparative and evolutionary neuroscientists will benefit, as we know very little about how the forebrain controls the response to psychological stressors in any animals other than a few rodents and primates. Knowing how evolutionarily conserved these mechanisms are or whether these connections have evolved several times in different lineages of animals, is interesting from the point of view of understanding brain evolution generally.

The other major group of people who will benefit are animal welfare scientists. The ultimate goal of this programme of work is to better understand how some animals may be more resilient to psychological stressors than other animals. Because this resilience is eventually going to come down to differences in how the forebrain regulates the stress response, understanding the forebrain regulation of the stress response is important. In addition, direct investigations of environmental manipulations of stress resilience will allow animal welfare scientists to design better rearing and housing conditions for commercial poultry.

Whenever possible, data and/or tissues can be shared with other researchers to reduce the use of animals.

And because of this, in the longer term, the chickens themselves (or at least future generations of chickens) will benefit from this research, if we can improve their resilience to stressors, and hence improve their welfare. Improved welfare can lead to improved yield (especially in egg production), as well as the possibility of marketing products as higher welfare, which will benefit the poultry industry.

How will you look to maximise the outputs of this work?

Study results will be published in technical journals and presented at scientific conferences. We will also write press releases about interesting findings for the general public. Any impacts on animal welfare, and on poultry practice, will be communicated to the poultry industry, policy makers, and/or other stakeholders.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 380

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Because we are ultimately interested in the welfare of laying hens and broiler chickens, we need to study the neural mechanisms of stress regulation in the brains of domestic poultry. For particular studies, we may need to study either laying hens or broilers (or both). We may study birds from pre- hatching to adulthood, depending on the exact question we're asking and the age of the environmental manipulations.

For environmental manipulations of stress resilience, we will use manipulations and birds that are appropriately matched to the commercial conditions in which the manipulations might be relevant, both in terms of age and strain. This could be before hatching, during early post-hatch life, or indeed at any stage of the life course that is relevant.

Typically, what will be done to an animal used in your project?

There are three typical routes an animal can go through in this licence:
Route 1 (involving brain surgery):

Animals will undergo brain surgery in which we (a) inject a tract tracer (i.e. a substance that allows us to identify which brain areas are connected to which other brain areas) into the brain, to map brain connections; or (b) inject a viral vector (a way to insert genes into cells) into the brain to express a receptor that responds to a specific designer drug (or a control injection); or (c) implant chronic guide cannulae; or (d) implant chronic electrodes into the brain. They may also receive a subcutaneous heart rate logger at the same time. The birds are then allowed to recover from this surgery for at least a week. Depending on the purpose of the study, what happens next varies. (a) if the bird received an injection of a tract tracer into the brain, it will just be kept alive under normal housing conditions after that until we collect the brain; (b) if the viral vector was injected, they will receive systemic injections with the designer drug (or vehicle) to activate the newly expressed receptors (and hence increase or decrease neuronal activity in the target neurons); (c) they will receive injections of a drug to temporarily inactivate neural activity (or vehicle) through the guide cannula; or (d) a light-weight logger will be attached to the implanted headstage to record electrophysiological activity. The bird could then be exposed to an acute stressor (e.g. 30 min in a bag, like Route 1) to assess the effect of the manipulation on the stress response. This exposure to an acute stressor can happen several times per day, depending on the experimental design. Blood may be sampled before, during and after the stressor (only one stressor, not all stressors if more than one per day). At the end of the study, the birds will be humanely killed to collect brain and other tissues. Depending on the study, this may involve perfusion fixation (i.e. running first a wash solution and then a fixation solution through the animal's blood vessels in order to fix the tissue from the inside out).

Route 2 (responses to stress):

Animals are sourced from different environments/early life conditions or they are exposed to environmental manipulations hypothesized to increase stress resilience for a number of weeks. They may or may not be implanted with a subcutaneous heart rate logger. Then, they will be exposed either to an acute stress experience (e.g. being held in a bag for 30 min) or a chronic stress experience (e.g. 8 weeks of unpredictable chronic mild stress (UCMS), which includes such mild stressors like mixing social flocks, temporarily removing food or enrichments, changes in the light cycle, etc). We may collect blood samples before, during and after the stressful experiences. At the end of the study, the birds may be killed humanely to collect brain and other tissues to identify brain areas that responded to the stressors and how strongly they responded. Depending on the study, this may involve perfusion fixation (i.e. running first a wash solution and then a fixation solution through the animal's blood vessels in order to fix the tissue from the inside out). If birds are not required for tissue collection, and they are deemed suitable by the NVS, they may also be rehomed.

Route 3 (tissue collection for ex vivo studies):

Animals are deeply anaesthetized and then killed while under anaesthesia to collect tissues. The protocol allows us to ask some anatomical questions without the animals needing to suffer at all.

What are the expected impacts and/or adverse effects for the animals during your project?

For the animals undergoing Route 1, the impact will always be of moderate severity, as the birds undergo a long (3-4 hours) surgery and have to recover from the anaesthesia and the surgery (including healing and the risks of infection; both will be managed with post-op steroids to suppress inflammation and antibiotics to prevent infection, when appropriate). The effects of the injections and of attaching the logger are mild, and the effects of the stressors are as in Route 2.

For the animals undergoing Route 2, the impacts and adverse effects will be moderate for most birds. The subcutaneous implant of the heart rate logger is a brief procedure (10 min or so), although it is performed under general anaesthesia. Blood sampling is mild, as it typically involves a brief prick of a vein (in wing or leg) and collection of blood using capillary tubes. The acute stressor is per definition stressful, but there is no lasting damage from this stressor. However, if a chronic stressor is used, this may have more adverse (psychological) effects on the animals, as it is designed to mimic long-term poor welfare conditions. A typical chronic stressor consists of individual stressors (e.g. food deprivation, wind, social isolation, disruption of the day-night cycle) presented to the birds once per day over a period of up to 10 weeks. In our experience, this is not severe enough to be visible in the birds' everyday behaviour, nor in their physical condition, although it is detectable at the level of some post- mortem brain markers. We will monitor flock behaviour and physical condition (body mass, feather condition) and stop the treatment if the effects are severe enough to affect the birds' physical condition or behaviour strongly enough, as indicated by our score sheets.

For Route 3, the animals do not suffer at all.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most animals will fall into the moderate severity category, either because of the surgery or because of the experience of an acute or chronic stressor. Some animals will fall in the mild category, as they may only experience blood sampling and humane killing to collect tissues. Animals in Route 3 fall under Non-Recovery.

What will happen to animals at the end of this project?

- Killed
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Because our questions are about the effect of the environment on the stress response of the animals, as well as about the brain mechanisms that control this stress response. Neither question can be answered without entire animals, as the brain is needed to respond to the environmental manipulations.

Which non-animal alternatives did you consider for use in this project?

Effects of the environment on stress responses can be studied in insects.

It is also possible to study neuronal connectivity in early embryos (before E14).

Why were they not suitable?

We are specifically interested in the regulation of the stress response and the effects of the environment on stress resilience in poultry. Insect physiology and behaviour is very different from poultry, and is therefore not appropriate to answer these more species-specific questions.

Although we could potentially do some of the tract tracing work in early embryos, the adult pathways that we are interested in do not develop until at least embryonic day 18, which is 3 days before hatching, and would fall under the Act. Depending on the connections we are interested in, we may work in newly hatched birds, juvenile birds, or adult birds.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These are estimated numbers, based on the experiments we have currently planned. As each individual experiment is planned, animal numbers will be reviewed and total number of animals may be revised accordingly.

Here is the breakdown per protocol:

Brain connectivity: 30 animals for tract tracing; this depends on the accuracy of our injections, but will need several animals per site to make sure we inject in the right place (due to inter-animal variation in anatomy)

Chronic brain implants: 20 animals - these within-animal comparisons require 6-10 animals per experiment to obtain significant results (depending on the effect size), so we have planned for 2 experiments

Manipulation of neurons: 30 animals: this takes about 10-12 animals per group, and you need two groups (there is a control group injected with a dummy virus); we have planned for one experiment, plus a few extra animals for pilot work on virus development.

Chronic stress studies: 100 animals: these will be typically 2x2 designs, requiring 12 animals per group, so 48 per study. We have estimated for 2 studies, with 4 animals to spare.

Acute stress studies: 100 animals: these will be typically 2x2 designs, requiring 12 animals per group, so 48 per study. We have estimated for 2 studies, with 4 animals to spare.

Tissue collection: 100 animals: in-vitro tract tracing in late embryos and early post-hatch chicks is a refinement method that we intend to implement. We have planned up to 100 birds from which tissue will be collected for this method.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Whenever possible, we will use within-subject designs. This is possible when studying the role of a particular neuronal population in controlling the stress response, as long as we use a reversible inactivation of that population (e.g. using a local inactivation using either virus-based technologies or guide cannulae to inject reversible toxins into the target population). Within-subject designs mean that the same animal undergoes all treatments, and the comparisons between treatments can be made on

an animal-by-animal basis. Because there usually are significant individual differences between animals, comparing within each animal increases the statistical power of the experiment and reduces the number of animals used.

When within-animal designs are not possible (e.g. rearing or environmental manipulations), we aim to reduce the extraneous individual variation by housing and treating the animals as similarly as possible, especially between the two groups being compared, such that only the manipulation under study differs between them. This will reduce the variation between birds in the outcomes of the study, leaving mostly variation that is due to our manipulation. This increases statistical power, which means we can use fewer animals to achieve the same outcome than if we had not controlled for these extraneous variables.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will conduct careful literature reviews to make sure we know what is already known, and will build on this knowledge constructively. When killing animals, we will collect all the tissues we could possibly need later, so we do not have to repeat experiments to measure different outcome variables. We have experience with these kinds of studies. However, when we use new techniques (e.g. the viral vector insertion of transgenes), we will first do pilot studies with a few animals to estimate the effect size, before designing the full study with the appropriate number of animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use domestic fowl. They are not models. They are the species we are interested in.

- The methods being used include:
- subcutaneous implant of heart rate monitors
- intracranial stereotaxic implants and/or injections
- blood sampling
- systemic drug injections
- stress induction
- environmental manipulations
- tissue collection for in-vitro work

Why can’t you use animals that are less sentient?

I have explained that animals at a more immature life stage cannot be used to trace the connections we’re interested in, until near hatching. At this point, chicks are completely independent, and should be considered just as sentient as adult chickens. We also cannot use other species, because our direct interest is in chickens. The ultimate application of this work will be to identify new chicken welfare measures and/or new manipulations that improve chicken stress resilience. Such mechanisms can be quite species specific, so need to be studied in the animals which we aim to ultimately benefit.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Every procedure will be evaluated and assessed for future refinements, and these will be implemented into the next procedures. If these are inconsistent with the current Project Licence, we will apply for an amendment. These refinements will be incorporated into our Standard Operating Procedures whenever implemented. We have a track record of refining our surgical procedures over the last PPL, including the use of sterile petroleum jelly to protect the brain from dental cement and the routine use of post-operative steroid to prevent seizures due to brain swelling after surgery. All these refinements have been made (and will be made) in collaboration with the veterinary team and the NTCO.

For anatomical tract tracing, we will use in-vitro tract tracing for shorter connections that can be traced in young animals (late embryo to early post-hatch) whenever possible, and only use stereotaxic injections in adult birds for later-developing or longer-distance projections.

We have developed a score sheet for monitoring the chickens' welfare and will continue to refine this score sheet.

			2c	3a	
Eyes	Open, normal 0	Not fully open 1	Half-closed +/- secretions 2	More than half closed or milky secretions 3a	
Faeces	Normal, fully formed and/or no faeces sticking to cloacal feathers 0	Normal but faecal matter evident on feathers 1	Diarrhoea 2b	Diarrhoea with blood 3a	
Plucked feathers	No evidence of feather plucking 0	New single patch < 1cm across with no skin damage/erythema 1a	>1 new patch and/or area increase >1-3cm affected +/- skin damage 2b	>3 patches >3cm with skin damage or bleeding 3a	
Respiration- when undisturbed Need a pre-procedure baseline if post-op	Respiratory rate within 15% of baseline (normal 15-40 rpm) 0	Resp rate 15-30% higher than baseline 1a	Resp rate 30-50% higher than baseline or any open beak breathing for >5sec, sneezing or head shaking 2b	Resp rate > 50% higher than baseline and/or nasal discharge 3a	
Surgical Wound (post-op only)	No swelling, heat, redness, discharge or pain 0	Swelling and/or redness but no discharge or pain 1	Swelling and/or redness with discharge and/or pain or wound open <50% 2b	Swelling, redness, discharge and/or pain with wound open >50% 3a	
				TOTAL SCORE =	

1a – increase monitoring

2a- give subcutaneous fluids, reassess in 1 hour, can be given up to 3 times then consult NACWO/VET

2b - consult NACWO/vet - interventions maybe available

2c –increase monitoring

3a-this finding may required humane killing, consult NAWCO/NVS

3b-arrange to humanely kill.

			2c	3a	
Eyes	Open, normal 0	Not fully open 1	Half-closed +/- secretions 2	More than half closed or milky secretions 3a	
Faeces	Normal, fully formed and/or no faeces sticking to cloacal feathers 0	Normal but faecal matter evident on feathers 1	Diarrhoea 2b	Diarrhoea with blood 3a	
Plucked feathers	No evidence of feather plucking 0	New single patch < 1cm across with no skin damage/erythema 1a	>1 new patch and/or area increase >1-3cm affected +/- skin damage 2b	>3 patches >3cm with skin damage or bleeding 3a	
Respiration- when undisturbed Need a pre-procedure baseline if post-op	Respiratory rate within 15% of baseline (normal 15-40 rpm) 0	Resp rate 15-30% higher than baseline 1a	Resp rate 30-50% higher than baseline or any open beak breathing for >5sec, sneezing or head shaking 2b	Resp rate > 50% higher than baseline and/or nasal discharge 3a	
Surgical Wound (post-op only)	No swelling, heat, redness, discharge or pain 0	Swelling and/or redness but no discharge or pain 1	Swelling and/or redness with discharge and/or pain or wound open <50% 2b	Swelling, redness, discharge and/or pain with wound open >50% 3a	
				TOTAL SCORE =	

1a – increase monitoring

2a- give subcutaneous fluids, reassess in 1 hour, can be given up to 3 times then consult NACWO/VET

2b - consult NACWO/vet - interventions maybe available

2c –increase monitoring

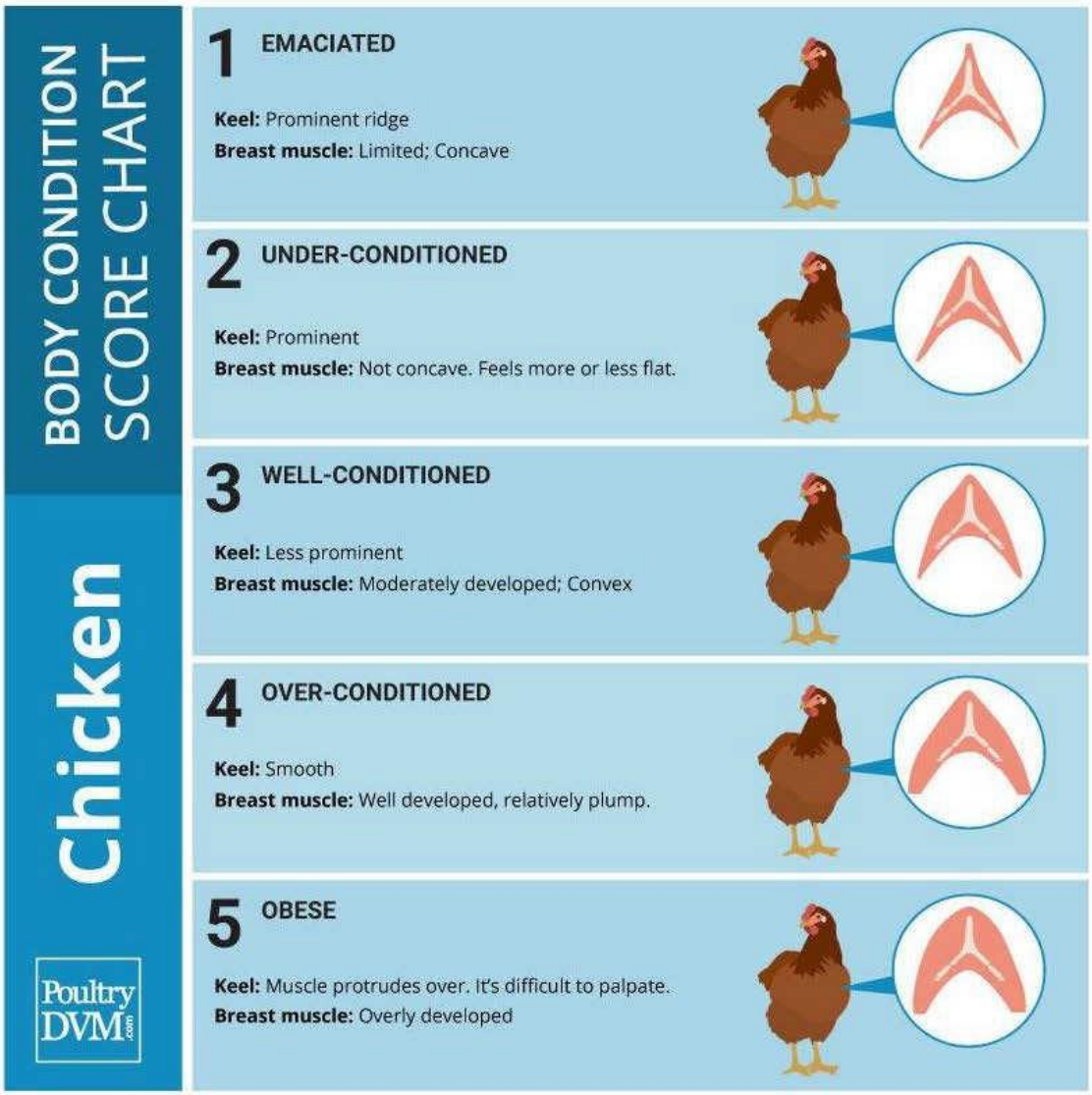
3a-this finding may required humane killing, consult NAWCO/NVS

3b-arrange to humanely kill.

*Body mass changes are estimates, as we don't have current data on how much body mass the birds typically lose after surgery and how long it takes them to stop losing weight. These numbers will be updated based on further data.

Score groups
0-4 Continue normal monitoring 5-10 Examine and rescore daily >10 Consult NACWO/NVS with view to humane killing >20 Humanely kill N.B. any animal scoring 2 in 3 or more characteristics consult NACWO/NVS

This is a document in progress. It will be updated as appropriate when new information comes available about monitoring the health of hens and broilers, following advice from NACWO and NVS.



What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the Code of Practice for Housing and Care of Animals Bred, Supplied, or used for Scientific Purposes (https://www.gov.uk/government/publications/code-of-practice-for-

the-housing- and-care-of-animals-bred-supplied-or-used-for-scientific-purposes), as well as the BVAAWF/FRAME/RSPCA/UFAW report on Laboratory Birds: Refinements in Husbandry and Procedures.

When these documents do not sufficiently cover a particular area, we will also consult other documents when appropriate, such as the CCAC guidelines on the care and use of farm animals in research, teaching and testing (Canada), ANZCCART's Fact Sheet on Domestic Chickens (Australia and New Zealand), and the Guide for the Care and Use of Agricultural Animals in Research and Teaching (USA).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

For more generic advances in the 3Rs, we will regularly look at the NC3Rs website for relevant information. We are also members of the Animal Welfare Research Network, and we will keep abreast of any new developments through their resources as well. We will also be in constant conversation with the veterinary staff, the NACWO, NVS and NTCO, to benefit from their knowledge.

In addition, we will keep abreast of the scientific literature on chicken welfare and procedures on birds, including unpublished results as presented at relevant scientific meetings.

181. Targeting Glucose Metabolism in Fatty Liver Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Liver disease, Chronic disease, Drug development, Therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To develop selective inhibitors of the liver enzyme glucokinase with high potency on the protein and good efficacy at the cellular level in isolated liver cells.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Fatty liver disease is the fastest growing cause of liver disease globally affecting 25% of adults globally. The early stages of this condition affect one in three adults in the UK. Depending on metabolic disease severity and genetic predisposition, fatty liver disease can progress from a mild condition to a more severe disease leading to liver inflammation and loss of function and ultimately liver failure.

There are no approved treatments and there is a major unmet medical need for drugs that will benefit people with genetic predispositions to liver disease.

What outputs do you think you will see at the end of this project?

In work leading up to this project we identified compounds that are the starting point for development into drugs for liver metabolism. In the current project these compounds will be further developed and optimized by making large numbers of modifications which we will test in normal mouse liver cells and in liver cells of a mouse that models a common genetic predisposition to liver disease in man (GCKR, rs 1260326). The mouse model for this condition will be bred specifically for this project. We expect by the end of the project to have identified a compound with high efficacy that can be used as a candidate drug for liver disease. We also expect to have better understanding of why liver cells with a specific genetic predisposition to liver disease respond differently from normal liver cells. Compound optimisation on isolated liver cells enables testing of large numbers of compound modifications under a range of conditions with relatively few numbers of mice (reduction and refinement).

Who or what will benefit from these outputs, and how?

Recently research groups working on diabetes have recognized the need to develop a new class of drugs that can protect from development of diabetes by targeting glucose metabolism. However, no such chemicals have been identified. The drug that we aim to develop in our project is expected to benefit both fatty liver disease and diabetes. Fatty liver disease occurs commonly in people with type 2 diabetes or who are overweight.We aim to generate a new-candidate drug that will be of benefit to researchers working on either fatty liver disease or type 2 diabetes to test its efficacy on protecting from both these common conditions. The ultimate goal is that the drug will be tested in human clinical trials for fatty liver disease and diabetes. The work will also advance knowledge of mechanisms linked to a common genetic predisposition to fatty liver disease. The results will be made available to the scientific community at scientific meetings and through publications.

How will you look to maximise the outputs of this work?

There are currently no drugs that selectively target the protein that is present in liver and in specialized sites in the body by a mechanism that slows its activity. this project is a collaboration between chemists who design and make the new chemicals and biochemists who test them for their efficacy in liver cells (normal and with genetic predisposition to liver disease). We are collaborating with "structural biologists" who will determine how each new drug identified interacts with its target. Work of this nature has not been done previously for the mechanism we are studying. It will therefore be "first-in-class" in terms of both target and disease state and will be made available to the scientific community through publication. The production of the new candidate drug would enable future collaboration for testing this in the disease state, with the ultimate objective of leading to a new therapy for fatty liver disease and type 2 diabetes.

Species and numbers of animals expected to be used

- Mice: 300 mice

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will be using a genetically altered mouse model that was designed to mimic a common human genetic predisposition to liver disease. We have studied the mouse model during the past 3 years and found that it has lower levels in the liver of two key proteins that are also lower in liver biopsies of people with the genetic predisposition to Fatty Liver disease. This mouse is healthy with no obvious signs of disease when maintained on healthy diets. Adult mice from this mouse model enable us to develop drugs that are effective in this genetic status. It also will provide new information on drugs that work differently in this genetic status which will improve our understanding of liver disease.

Typically, what will be done to an animal used in your project?

An animal will typically be bred using standard breeding practices and genotyped, usually once, using appropriate and least invasive methods such as ear biopsy. They will then be subjected to a non- recovery procedure involving total anaesthesia followed by administration of heparin intraperitoneally while under anaesthesia followed by laparotomy, cannulation of the liver and exsanguination under terminal anaesthesia The project essentially involves the testing of our chemicals on liver cells prepared from adult mice that have either the genetic predisposition (Gckr:P446>L) or are normal. (Gckr:446P, e.g. C57BL6 wild-type).

What are the expected impacts and/or adverse effects for the animals during your project?

Based on our past experience with this genetic mouse model we do not expect the mice to show any signs of stress or disease as a result of the genetic status because they will be maintained on diets which do not cause metabolic stress. The assessment of genetic status from an ear biopsy may cause brief and mild stress.

The procedure for obtaining liver cells is under terminal anaesthesia and involves exsanguination during perfusion of the liver.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We estimate that we need to assess the genetic status by tissue biopsy on approximately half the mice that will be bred (~150), mainly those mice used for breeding stock only, as the remaining experimental mice will be bred by homozygous pairs for either the major allele (equivalent to wild-type) or minor allele (equivalent to genetic risk model). The severity of tissue sampling is mild.

We will be using 160 of the mice for preparing liver cells by the non-recovery procedure under terminal anaesthesia.

Mice not used for liver cell preparation will be killed humanely by schedule 1 methods.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are developing a new drug that interacts with an enzyme that is expressed mainly in the liver and to a lesser extent in specialized tissues in the body. The efficacy of the drug in liver cells is critically dependent not only on the presence of the target enzyme but also on the "interacting partner proteins" and their relative abundance. Our recent work has shown that small differences in the relative abundance of the target enzyme and partner proteins can markedly affect the response to the drug.
There are no liver-derived cell lines that express both the target enzyme and the partner proteins in similar amounts as in either normal liver or livers of the common genetic predisposition to liver disease. Liver cells from normal mice and the genetic predisposition to liver disease are essential to test drug efficacy.

Which non-animal alternatives did you consider for use in this project?

The target enzyme but not specific partner proteins is expressed in a non-liver cell line which will be used in our project. From the identification of the 3 starting compounds from our previous project, we identified a non-liver cell line that expresses the target enzyme (but not liver-specific partner proteins) that is more sensitive to the drugs than the liver cells. Our compounds will first be screened through this model to enable us to test only the more active compounds in liver cells. This minimizes the number of experiments that need to be done on liver cells.

Why were they not suitable?

Our experience from testing various cell lines is that ones that express the key target enzyme but not the partner proteins do not show the same drug efficacy as liver cells that are prepared from the mice. We are therefore reliant on studies not only on liver cells derived from mice but also on the normal compared with genetic predisposition, because drug efficacy is critically dependent not only on the level of the target enzyme but also the relative abundance of the partner proteins.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We estimated the number of animals we will use from the number of chemical structures that will be synthesized for each of the chemical series on the assumption that half of these will need to be studied in liver cells after testing in the cell line.

We estimated the number of animals based on the number of experiments that will be required to be confident that differences in genetic status are real and not generated by chance.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use a breeding strategy to ensure efficient breeding fit for purpose: i) for colony maintenance we will breed heterozygous mice; ii) for liver cell experiments we will breed homozygous mice for either normal genetic status (Gckr446P; WT) or genetic predisposition (homozygous Gckr446L) separately for each case.

We will optimise for maximum number of compounds for each experimental round to minimize the number of experimental rounds as far as is practicable without compromising data quality. In our assessment cascade, we have built in in vitro (enzyme assays) at the beginning to reduce the number of compounds that would need to be tested in cells.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use statistical analysis to determine the required number of experiments to generate scientifically robust and valid conclusions.

We will use male and female mice homozygous for either the major (equivalent to wild-type) or minor allele to assess differences in response linked to genetic status. From compounds of high potency we will be able to determine whether differences linked to genetic status manifest similarly in male or female mice.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use a genetically altered mouse model with a minor change in one gene expressed in the liver. This model mimics a human genetic variation that affects a high proportion of people (1 in 3) and as expected the mouse model does not show any evidence of pain, suffering, distress or harm on a normal diet compared with normal mice.

The procedure for isolation of liver cells is performed under terminal anaesthesia and the mice do not respond to the anaesthesia differently from normal mice. This mouse model is

essential to the project as no liver-derived cell line is available as an alternative for testing these compounds.

Why can't you use animals that are less sentient?

The gene responsible for common genetic predisposition to liver disease is present in lower animals. However, the specific gene region involved in the genetic variation in human populations is the same (conserved) in mouse but not in less sentient animals. Lower vertebrates that are less sentient than the mouse cannot be used to model either normal human liver (which is critically dependent on small variation in the gene) or the common genetic predisposition to liver disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In order to avoid overbreeding, animals for hepatocyte isolations will be bred from homozygous breeding pairs. Only the first generation will be assessed for genetic status to minimise the number of mice experiencing mild discomfort.

Heterozygous breeding trios will be mated every 12 wks to 1) keep the mouse line alive, 2) replace homozygous breeding pair breeders every 9 months, and 3) occasionally provide mice for hepatocyte isolations as necessary. Mating trios can be reduced to pairs should any surplus of animals accumulate at any point during the project.

Our liver cell preparation has been extensively refined over the years. However, we will consider new advances through publications in the field and through 3Rs seminars.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Alongside the guidelines listed below, we will also adhere to local AWERB standards for research animals, and where appropriate, support the development of new local standards for refinements discovered during the project licence.

Code of Practice for Housing and Care of Animals Bred, Supplied or used for scientific purposes LASA Guidelines
RSPCA Animals in Science guidelines UFAW Guidelines and Publications NC3R's and Procedures with Care
We will consult with the Colony Manager to review genetic health, breeding practices and overall colony health and management at regular intervals.

We routinely keep abreast with modifications to the procedure on liver cell isolation from publications in peer reviewed journals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The local AWERB, NIO, NACWO, NTCO and Veterinary team regularly inform, and disseminate improvements and recent studies involving reduction, replacement and refinement

Alongside external resources including (but not limited to); collaborators, peers, conferences and lab animal and animal welfare bodies.

During the 1, 3 and 5 year review of the project licence I will update on implementation or consideration of the 3Rs that has occurred during the previous period, alongside a review of the linked training plan, score sheets etc. in collaboration with the NACWO, NTCO and Veterinary team with a particular focus on refinements.

182. Neuroimmune Influences on CNS Health, Injury and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Neuroimmunology, Brain injury, Cerebrovascular disease, Dementia, Microglia

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo, aged
Rats	neonate, embryo, adult, pregnant, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to identify interactions between the brain and immune system that influence brain health and responses to brain disease and injury. These interactions will then be manipulated to seek treatments that enhance brain health, resistance to disease, and recovery from injury.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Disorders of the brain are major causes of death, disability, and altered cognitive function (e.g. memory, emotions, how we perceive the world). Very few effective treatments exist for any brain disorder. We and others have shown in recent years that interactions between the brain and immune system may be critical for how the brain develops and its ability to resist and respond to brain disease or acute injury. Understanding more about these interactions is anticipated to identify new treatment targets that could enhance brain health, resilience and recovery.

What outputs do you think you will see at the end of this project?

We anticipate the key outcomes and benefits, achievable during the lifetime of the project, will include the following:

Advance in scientific understanding – the project will produce data providing new knowledge on how the immune system influences brain development and susceptibility/responses to brain injury and degenerative disease.

Identification of therapeutic targets – in further understanding mechanisms by which the immune system influences CNS health, injury and disease, we expect to uncover new targets for therapeutic intervention to alleviate CNS damage, promote repair and prevent complications, as our previous studies have done.

Proof-of-concept/validation of putative therapeutic agents – our previous studies have identified agents targeting neuroimmune pathways with the potential to enhance resilience to degeneration and repair of brain damage. Some of the work in this project will provide the data necessary to understand the potential to move candidate treatments towards studies in humans.

Methodology and technology development and refinement – we expect that, as our previous studies have done, our project will produce refinements in protocols that minimise potential

adverse effects in animals and also developments in techniques providing greater insight to neuroimmune biology e.g. development of new methods to assess immune cell function in damaged brains.

As an indication, during our preceding PPL from which this project evolves, key outputs included the following:

Disseminated new data and knowledge through 23 peer-reviewed manuscripts, three publicly accessible large datasets, >50 presentations at scientific meetings and >20 invited seminars at academic institutions and companies.

Identified three new targets for intervention including agents to modify them and preliminary data on proof-of-concept.

Established collaborations with three pharmaceutical companies supporting research into the above targets.

Influenced policy through participation in advisory groups resulting in publications describing best practice in preclinical modelling of human disease.

Who or what will benefit from these outputs, and how?

The key beneficiaries, in addition to my research group, will include:

Researchers and data analysts – researchers working in various disciplines (e.g. neuroscience, immunology, neuordegenerative disease, stroke) will benefit from the data, information and knowledge our project will generate. This can help inform the development of their own studies. Increasingly, we produce large datasets that can be mined by data analysts working independently from us, leading to further knowledge obtained from our source data.

Health professionals and healthcare systems – it is possible our work in this project will lead to changes in clinical practice within the lifetime of the project, although we expect the more likely impact is longer-term through our data informing decisions on progression of potential therapeutic agents towards clinical trials.

Industry – we expect our data will be used by companies to inform development of therapeutic approaches. We have existing formal collaborations with several pharmaceutical companies that offer routes to translation and may lead to intellectual property applications/agreements.

Ethics and welfare policy practitioners – information generated on refinements to protocols can be used to improve animal welfare.

Public - beyond the lifetime of this project our ultimate goal is to develop new approaches to treating patients with brain injury and disease in order to reduce death, disability and other complications caused by these disorders. We are realistic to understand this can usually only be achieved in the medium/long-term, however the work in this project will provide a vital step to realising these longer- term benefits.

How will you look to maximise the outputs of this work?

We will disseminate our research findings as widely and as promptly as possible so that the above groups can benefit. This includes through peer-reviewed publications, conference and meeting presentations, invited seminars, and attendance at advisory groups. We publish in open-access journals and make data freely available in open-access repositories so that others can use these source data without restriction. We have excellent existing collaborations with many other groups internally and externally that includes clinical colleagues with whom we regularly share information on ongoing projects. We also have ongoing engagement with several pharmaceutical companies through formal collaboration agreements. We participate in public outreach activities including educational activities with school-children so that awareness of our work and more general research progress can extend beyond the academic and clinical environment.

Species and numbers of animals expected to be used

- Mice: 4000
- Rats: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

Almost all studies will use mice. Although not identical, mice have many things in common with humans in the way that their brain and immune cells function that means information from mice can be used to predict how the same processes work in humans, particularly as we can use human samples to corroborate findings in mice.

Of species available for preclinical research, the breadth and depth of knowledge on nervous system and immune system anatomy, physiology and cell composition and function (e.g. through community- wide initiatives such as the Allen Brain Atlas and Immgen) is much greater for mice than any other species. This provides the most thorough framework in which we can contextualise new data and knowledge to maximise our understanding and the impact from it. The availability of research tools and methods for data collection is also most extensive and advanced for mice, particularly for measuring and manipulating immune cells - this enables richer understanding from fewer animals because multiple data types can often be acquired in the same experiment, which is much more challenging to do in other species.

We will study mice at early postnatal, adult and older ages because we want to understand processes that influence brain development, adult homeostasis and age-related disease. Many processes are connected across life-course phases and resilience to disease is likely influenced by brain function much earlier in life. There are also developmental processes that may be aberrantly reactivated later in life and so understanding early life mechanisms can inform how we interpret findings at older ages,

and vice versa e.g. there are striking similarities between the way some of the brain's macrophages (called "microglia") change their expression of genes during development and disease in ageing.

We will use a small number of rats to isolate brain cells for studying the cells in a dish ("in vitro"). The larger rat brain enables us to isolate more cells from each brain which can be important for studying rare cell types and therefore reducing the number of animals needed. Rat and mouse cells can also be mixed and the different species of origin enables us to distinguish cell source in certain types of analysis e.g. to understand how different brain cells communicate with one another.

Typically, what will be done to an animal used in your project?

The core of most studies will involve the modelling of aspects of human brain injury or disease, including stroke and degenerative brain disease - this may be a known genetic cause, a known/suspected pathophysiological mechanism and/or a pathological hallmark. We will achieve this using the following most common approaches:

a mouse containing a genetic alteration (produced separately to this project) equivalent to the human alteration.

a surgical (e.g. occlusion of a brain artery) or non-invasive procedure (e.g. exposure to a low oxygen environment) conducted on a mouse (with or without a genetic alteration) to mimic a pathological event contributing to injury/disease in humans.

In most studies, the above core methods will be combined with other optional procedures (usually a maximum of two) used to enable in-life data collection (e.g. assessing mouse behaviour or imaging of the brain by MRI) or manipulation of biological targets (e.g. injection of a drug into the blood or tissue). Often, this manipulation will be intended to test the potential benefit of a candidate therapeutic agent that, if successful, may be take forward to trials in humans.

Duration of experiments will depend on the specific scientific objectives of the study. For studies involving acute brain injury, the mice are typically studied for no longer than two weeks after injury, very rarely for up to 6 months to study long-term repair. For studies modelling chronic disease, experiments will typically age mice from birth until 3-12 months old.

What are the expected impacts and/or adverse effects for the animals during your project?

For many procedures, there is likely to be only a transient impact on the animal and a rapid return to normal behaviour without any intervention e.g. after blood sampling, drug injections, brain scanning.

For surgical procedures and those causing brain injury/disease there is the potential for the following: Change in eating and drinking habits
Weight loss

Signs of neurological injury e.g. limb weakness, movement coordination impairments, memory problems

The duration and frequency of these will depend on the individual experiment but it is expected that weight loss and eating/drinking habits will be commonly observed and recover within a few days. Signs of neurological damage may persist permanently (as they often do in humans), necessary in a minority of studies to study long-term consequences of disease and the effects of candidate treatments to assess if any benefits are sustained. These neurological deficits, if they do occur, do not normally adversely affect the normal day-to-day behaviour of the animals more than transiently even if specific sensitive neurological tests can detect deficits over a longer period.

Pain is a possible adverse effect associated with tissue damage during surgery (NB brain damage itself is not painful as there are no pain sensors in the brain) however this will be limited in extent and duration by giving analgesic drugs prior to and/or after surgery.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Overall, we anticipate that ~40% of animals will experience no more than mild and transient discomfort (or none at all). The remainder of animals may experience up to a moderate severity. This is because around one third of the animals used in most surgery-based studies will be control animals not exposed to surgery or carrying disease-causing genetic alterations. In studies not involving surgical/invasive procedures, control animals comprising around half of animals will typically experience no more than mild discomfort.

The remaining animals will be exposed to no more than moderate degree severity due to model/procedure refinements and control measures in place.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Achieving our project aims requires understanding processes that involve interactions between complex body systems, particularly the nervous system, immune system and cardiovascular system. To make best advances in knowledge, some studies need these interactions intact in living organisms as they are far too complex and incompletely understood to model by computational methods alone. Animal models, used appropriately and as part of a multi-method approach, enable us to gain more precision and depth in the understanding we generate compared to solely non-animal approaches and are likely to produce faster and more successful translation of treatments to humans. Almost all our studies involving live animals will use mice - we need a mammalian system with a complex nervous system to best model humans so non-animal species and invertebrate species are very limited in suitability.

Which non-animal alternatives did you consider for use in this project?

We have considered several non-animal alternative methods, all of which will be used to complement the animal studies in our overall project, but alone would provide only limited understanding far less likely to lead to benefits in humans:

Human tissue samples and brain scans - includes post-mortem brain tissue from donors held in brain banks, blood samples, brain scans (e.g. CT, MRI)

In vitro cell culture - brain and immune cells derived from sustainable cell lines that can be grown cultured in a dish, including individual cell types in isolation or more complex mixtures that form organ-like structures called "organoids"

In silico analysis - includes use of computer models of real data and re-analysis of large datasets from public databases

Why were they not suitable?

While each non-animal alternative has utility, there are also several limitations:

- Human tissue samples and imaging - brain tissue is usually only obtainable at post-mortem (very rarely as biopsy tissue) so mostly provides information on end-stage disease, sometime many years after disease-causing processes start i.e. consequences rather than causes of disease.

- While analysis of brain scans and blood samples in-life can be informative, in isolation, they cannot adequately reveal the details of molecular and cellular processes within tissues.

- In vitro cell culture - cell cultures (either of individual cells or more complex mixtures that can form organ-like structures) cannot adequately mimic the highly complex body-wide interactions between the nervous system, immune system, and blood vessels

- In silico analysis - computer models are not yet capable of providing sufficiently accurate predictions of the complex interactions among the body systems we need to study to achieve our aims, in part because of limited data to build the computer models to begin with (itself often derived from animal studies). The increasing availability of large datasets in public repositories is helpful for some studies as new insight from re-analysis is possible, however this cannot replace methods that enable manipulation of novel targets and mechanisms to establish if there are causal functions or if new candidate treatments are effective.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Total animal numbers are estimated based on projections for numbers of experimental animals needed for individual studies combined with the range of studies we anticipate will address our overall aim over the project period, in so far as it is possible to judge that over a long-term period (5y) where we have to be responsive to unanticipated developments and changing practices. These numbers also account for breeding and maintenance of rodent colonies to generate experimental animals. The total numbers are in line with our use of animals over previous licence periods (e.g. as included in Home Office annual returns) - while our experimental objectives evolve from previous work, the range and scale of studies is likely to be similar hence we expect to use broadly similar numbers in future.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have applied good principles of experimental design following guidelines provided by organisations such as NC3Rs and from our own involvement in advisory groups around best practice in studies using animals. As part of this we can formally estimate the optimal numbers of animals likely to enable us to address specific scientific questions that account for the expected variability in data (based on previously acquired data of similar type) and the size of any differences between experimental groups (e.g. drug versus control treatment) that we consider biologically meaningful. This ensures we do not use more animals than necessary to make important new findings. We have also considered the optimal use of control groups such that in some studies a separate control group is not required because the same animal can act as its own control (e.g. assessment of behaviour before and after administration of a treatment or induction of brain injury) - this

can reduce the number of experimental groups and therefore the number of total animals needed in a study without compromising our ability to detect biological effects.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Other measures we use to reduce total numbers of animals include:

- designing efficient breeding strategies so that the greatest proportion of offspring carry the desired genetic make-up required for use in experiments
- achieving some parts of data analysis from tissue derived from animals shared by multiple labs with complementary but distinct objectives
- ensuring best practices in animal husbandry and housing including consistency and stability of environment, handling, and monitoring to minimise variability that would artificially inflate the number of animals needed to detect biological effects
- use of validated protocols that minimise introduction of avoidable "noise" variables that would artificially inflate the number of animals needed to detect biological effects
- using pilot studies with very small numbers of animals initially where a less established or standardised protocol is required so that feasibility and best practice can be ensured before proceeding to more definitive and substantive experimental studies

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The models and methods we use mostly comprise the following:

Models of CNS injury and disease - we will use animal models of human brain vascular and degenerative disease. Most models are well established and accepted in the field and chosen to best enable our scientific objectives to be met while achieving the most refined approach. For acute injury models, we limit the extent of brain injury to no more than is necessary to understand how the damage is caused or how it can be repaired and minimise the recovery time after injury. For modelling more protracted disease in humans, we are usually interested to understand the early events leading to disease therefore we can conduct most studies in the absence of or prior to disease signs appearing. We may incorporate use of less sentient early larval zebrafish forms (prior to free feeding stage) in some studies where these can be adequately informative after accounting for differences with mammalian species.

Methods to measure biological variables and enable data acquisition - these include a range of optional procedures such as brain scanning, observation/measurement of animal behaviour, and blood sampling. Many of these can be achieved non-invasively and for any invasive techniques we follow up-to-date guidelines to minimise risk of distress or adverse effects.

Methods to manipulate biological targets - this generally involves the administration of substances such as a drug to block or stimulate a biological pathway. We always choose the least invasive approach to administer agents that can achieve the required localisation or distribution of the substance within the body.

Why can't you use animals that are less sentient?

Our aim is to understand how interactions between the nervous and immune system influence brain health and disease/injury responses therefore it is important we use animals that have these complex system interactions - rodent species enable this whereas this is not achievable using invertebrate animals, some of which lack key immune cells involved in brain function. While zebrafish can model certain neuroimmune features relevant to human, notable differences have been reported that suggest less similarity than rodents to humans.

Some of our studies also require a functioning cardiovascular system with circulatory dynamics similar to humans (e.g. to model stroke in humans) that generally are only possible to achieve using mammalian species such as mice. Movement of immune cells around the body through the circulation is a key part of the body's immune response, including after brain injury and during disease.

Many studies will assess aspects of cognitive behavioural function which requires species with sufficient neurophysiological complexity to produce behavioural patterns relevant to human cognitive impairments - mammalian models are best suited to this requirement.

While we will perform some protocols under terminal anaesthesia, it is not possible to conduct behavioural assessments and studying more chronic processes influencing the course of brain disease or recovery from brain injury is not feasible under prolonged anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will refine basic practices for housing and handling animals in line with up-to-date guidelines (e.g. from NCR3s) to ensure animals are always maintained with the least risk of distress. This will include appropriately enriched housing environment and handling carried out by trained staff only.

All animals are monitored regularly by experimental and/or facility staff, with frequency and type of observations tailored for animal age and scientific protocols being used, taking account of potential harms (e.g. if studies are conducted using older animals with increased risk of age-related morbidity, we apply tailored monitoring based on local guidelines for monitoring ageing rodents). Appropriate monitoring can identify potential for adverse effects before they happen, enabling adjustments to monitoring and prompt action to prevent these happening.

We follow guidelines such as IMPROVE and from NC3Rs to achieve best practice for pre- and post- operative care when using surgical procedures. Pain management is applied following consultation with veterinary staff at experimental design stage to ensure optimal approaches are used.

The use of strict, well-defined humane endpoints will be applied in all studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Routes and volumes for administration of substances are taken from Laboratory Animal Science Association good practice guidelines: administration of substances 1998 (http://www.procedureswithcare.org.uk/lasa_administration.pdf).

Surgery will be undertaken as per the Laboratory Animal Science Association Guiding Principles for Preparing for and Undertaking Aseptic Surgery 2017 (http://www.lasa.co.uk/wp- content/uploads/2017/04/Aseptic-surgery-final.pdf)

Standard operation procedures for most tests have been generated using data and expertise from multiple animal houses and can be found at https://www.mousephenotype.org/impress

Research community guidelines on experimental planning design, conduct, and reporting (e.g. ARRIVE, PREPARE and IMPROVE guidelines), some of which we have contributed to ourselves, will be consulted and followed as much as is relevant and practicable to do in each of our studies.

We also follow local institutional guidelines on husbandry, imaging, substance administration, blood sampling, and ageing to ensure studies incorporate best local practice on refinements.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay up to date with the NC3Rs literature and recommendations, through the NC3Rs newsletter, website and communications with staff. Will also be informed from regular communication with staff within our institution's research animal services department, including attending their compulsory bi-annual update for all licence holders. We maintain regular contact with veterinary and animal services staff and can implement any advances promptly into housing, husbandry and experimental design.

183. Investigating the Effects of Genome Instability on Tumour Development over Time

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Tumour evolution, intratumour heterogeneity, tumour initiation, resistance, therapy

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This Project Licence aims to investigate internal and external factors that affect cancer formation and shape the growth and the development of therapy resistance. We further aim to investigate the function of these identified factors in tumour development and in the normal development of tissues and organs. This will be done in order to not only predict therapy response and disease progression, but to ultimately control how tumours evolve to be able to channel them towards pathways that give better disease outcomes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Analyses of cancers have revealed evidence for ongoing genetic evolution and intratumour heterogeneity (ITH), a phenomenon where one cell within the tumour is genetically different from its neighbouring cell. This heterogeneity is generated by multiple factors and contributes to therapy resistance, which ultimately leads to disease relapse and progression.

The research of the laboratory integrates clinical, genomic and basic science data to understand how internal factors, such as the immune system or genomic instability, as well as external factors, such as environmental exposures (pollution, microplastics, substances found in tobacco smoke) or cancer therapy, shape the formation and evolution of cancers over time.

In order for us to understand the complex cross talk occurring between the evolving tumour and the rest of the body, we need to use animal models. We will generate animals that exhibit the same kind of genetic instability and evolution observed in human diseases and study the role of genetic instability in multiple cancer types including lung-, colorectal- and brain-cancers. The development of these cancers is poorly understood, and they all have a very poor prognosis when diagnosed in late stages. By understanding how cancers evolve, we aim to not only predict therapy response and disease progression, but to ultimately control these changes in order to channel the tumours towards a path that gives better disease outcome.

Some genes that are commonly lost in cancer also have critical roles in normal embryonic development. By investigating the function of these genes during development, we will gain important insights not only into the role these genes play in embryonic development, but also how their function is subverted in cancer. Increasing our general understanding of embryonic development may not only lead to new treatment strategies for cancers but also for the onset of congenital disorders involving these genes.

What outputs do you think you will see at the end of this project?

This project could answer key questions about tumour growth, spread and treatment response, laying the foundation for potential novel treatments, personalized cancer therapies and improving patient outcomes.

1. We expect the animal models used in this project to generate new insights regarding the development of cancer and the fundamental biological processes involved in normal development.

2. We aim to uncover potential cancer vulnerabilities which will help us in the design of novel anti- cancer therapeutic strategies.

3. We aim to understand the impact of the environment, especially air pollution on tumour formation and development.

4. At the end of the studies supported by this licence, the results will be made publicly available by scientific publications and presentations at conferences, thereby increasing general knowledge.

Who or what will benefit from these outputs, and how?

Within the duration of this licence, we expect short term benefits such as the publication of discoveries in scientific journals and at conferences. We also expect that the novel models of tumour disease that are generated will be of use for the wider scientific community.

As all the studies supported by this licence fall within the category of basic research, we expect that there mostly will be longer-term benefits. The knowledge gained will be used in further studies to investigate new cancer treatments and cancer risks. This project could answer key questions about cancer initiation, growth, spread and treatment response, laying the foundation for potential novel treatments, personalized cancer therapies and improving patient outcomes. Our research regarding air-pollution and cancer development might inform governmental policy decisions.

How will you look to maximise the outputs of this work?

In order to maximise the output of this work, we will not only publish our results in scientific journals, but also engage in public outreach to increase cancer awareness. In addition, we collaborate with pharmaceutical companies in order to improve cancer treatment. An important aspect of our work are the new animal models generated and we are collaborating with multiple laboratories in the UK and internationally to maximise the knowledge gained from our models and experiments.

Species and numbers of animals expected to be used

- Mice: 25 000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The area of research covered in this project licence focuses on the initiation and development of cancer and the genes involved in these processes. Work with mouse embryos is required to investigate the effects of the genes we are studying on normal development. Cancer is a disease that can affect an individual at all life stages and therefore juvenile, adult and aging mice are necessary to be able to model cancer development as closely as possible. We will therefore use all life stages of mice in this project.

The reason why we use mice and the life stages used in this research are the following;

-The studies and experiments in this project track living, evolving cancer cells as the disease progresses and changes in response to cancer therapies. Investigating the changes in the tumours and manipulating them through genetic engineering or therapeutic intervention, precludes the use of human volunteers or lower models such as zebrafish, and requires adult animals with immune systems that can be modified. Although the life span of a mouse is vastly different from a human, the processes involved in cancer formation and evolution are very similar in suitable models.

- There are many model systems and reagents already available for investigating multiple processes in mice. We can investigate the effects of, for example, the immune system on

tumour development by generic manipulations, additions of reagents developed for mice or by using readily available established genetic strains.

Typically, what will be done to an animal used in your project?

We are investigating the same fundamental biological aspects, i.e. tumour formation, progression and response to cancer therapies in multiple disease models such as lung, colon and brain. We are also investigating the effects of tumour promoting genes on normal embryonic development. In these experiments embryos are investigated at different developmental stages.

When investigating tumour formation and evolution, animals will form tumours, either spontaneously, through genetic modifications or via transplanted tumour cells or cell lines. When studying brain tumour formation, we implant brain tumour cells via a surgical procedure into the mouse brain. In some instances, we will investigate the effect of treatments (such as chemotherapy, immunotherapy or targeted therapy) on tumour development and on how drug resistance develops. We are also investigating how external factors such as air pollution impact the formation of cancer.

The lengthiest experiments performed in this project will be investigating the dynamics of tumour growth during disease progression, sometimes while on therapy, which depending on tumour model, can come close to the lifespan of the mouse. We always aim for all our experiments to last the minimum time required to answer the specific scientific question being asked, but due to the varied cancer types and models studied, the duration of the experiments varies tremendously; in some of the colon models, tumours form slowly and only become apparent after a year or more. In contrast, when investigating fast growing tumours or investigating early events in tumour formation, the duration of the experiment might be as short as 48 hours.

Where appropriate, we may monitor disease progression over time using non-invasive methods such as caliper measurements of superficial tumours or by using imaging methods such as computerized tomography (CT) scanning. In all our experiments we aim to use the least invasive methods possible for all reagent administrations such as intranasal administration or via oral gavage.

What are the expected impacts and/or adverse effects for the animals during your project?

In the parts of our projects focusing on tumour development, all experimental animals will experience a challenge, either external or due to a genetic modification. The main adverse effect will be tumour burden or emphysema, a lung condition that leads to shortness of breath and reduced lung function.
Depending on where the tumour is located the adverse effects will vary. Mice with lung tumours or emphysema will develop shortness of breath. If the mice have colon tumours, they will experience a maximum 20% weight loss, whereas mice with brain tumours will exhibit abnormal behaviour. The mice will only experience these clinical symptoms for a very limited period of time as all of these signs are clinical endpoints of the experiments.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

This licence contains multiple protocols where some are of mild (ca 70% of all animals within this licence) and some have moderate severity. For protocols where the aim is to investigate tumour growth, we expect a majority (80%) to reach moderate severity due to surgery, repeated procedures or tumour burden. We expect that the final 20% of the animals within the moderate protocols will never reach moderate severity. In all cases, we aim to limit the number of animals and the severity of procedures in accordance with the 3R's.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The studies and experiments in this project track living, evolving cancer cells as the disease progresses and changes in response to time, cancer therapies, environmental exposures or immune modulation. Investigating the changes in the tumours and manipulating them through genetic

engineering or therapeutic intervention, together with the fact that we predominantly are studying lung tumours, precludes the use of human volunteers or lower animal models, such as zebrafish.

Which non-animal alternatives did you consider for use in this project?

We complement the animal studies with cell models and cells from the mice as well as data obtained from patients in clinical trials. Where possible, in vitro experiments are performed to minimize the use of animals. For instance, cells taken from genetically modified mice are used to investigate identified cancer protein function in detail. We are currently developing a computational model to study the viability of cells after a whole genome doubling event and the effects of chromosomal instability on tumour development in general. This approach will complement our mouse experiments and will likely reduce the number of animals required for our studies. We are also planning to use knowledge gained using human tumour- and immune- cell material to develop similar model systems using mouse cells, which will reduce the number of animals needed to complete our studies.

In general, for the studies described in this licence, we expect 60% of the data to be generated by the use of animals, 30% to be generated from our clinical studies of patients with lung or brain cancer as well as data from the literature (colon). The final 10% of the data will be generated by work performed on cell lines.

Why were they not suitable?

Although cell models and patient cohorts will be used based on their specific strengths, we cannot yet model the complex interactions between different cell types that occur during cancer development or normal embryonic development outside of a living organism and therefore require the use of mouse models as well.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the numbers of animals used within this licence based on the ongoing projects, the number of people working within this licence and previous experience of animals required for projects of this scale. We are increasing the size of the group and a larger proportion of the laboratory will be doing animal research. Although the total number of animals is large, we are currently actively breeding 52 different genetically modified mouse strains.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In order to use as few animals as possible, we always use the same general strategies at the start of each experiment.

For all our experiments we search the literature and/or perform an initial, small-scale study to investigate the effect size of the treatment or genetic modification we are investigating. Data from previous, similar experiments, by our laboratory and by others, are also used as a guideline. These results are then used to decide the number of animals required for the study using power calculations or the NC3R's Experimental Design Assistant. Trained statisticians are also available if additional help is needed in this regard.

Individual experiments involve planning and consultation within our team, colleagues and external collaborators. Experimental strategies are analysed before, during and after the experiments, to refine and reduce animal numbers if possible. For example, data from one experiment might inform and reduce the number of animals in subsequent experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In order to reduce the number of animals used, we strive to maximize the data generated by each animal, i.e. multiple different experiments use tissues from the same animal. This is done both within the group, and where possible, in collaboration with other research groups. To further increase the possibility of tissue sharing, we are aiming to set up a mouse tissue biobank.

Where possible, mouse strains are bred to homozygosity in order to reduce the number of animals born with unusable genotypes. In these cases, every animal born for the experiment can be used, which reduces the number of animals required, particularly for

complex genotypes, leading to reduction of animal usage. Cryopreservation of gametes, embryos, tissues and cells is routine at our establishment and will ensure that the minimum number of mice is bred.

We also reduce the biological variation within the experiment by, where possible, using littermates as parents to generate the experimental animals, thereby limiting genetic differences, which ultimately leads to fewer animals required to reach a reproducible conclusion. Experimental bias will be further reduced by blinding the genotypes to the lab/research staff who measure outcomes.

For each experiment, tumours will be induced in age and sex matched mice, thereby reducing the variations within the experiments and as a result minimize the number of animals needed. Where feasible, we aim to generate cell lines, histological-tissue surveys and other resource archives in anticipation of downstream and future analyses.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this project we are using genetically engineered mouse models as well as transplantation models of cancer. We are currently investigating tumours originating in the lung, colon and brain. The genetically engineered mouse models we use generally do not lead to any harm to the animals before tumour induction. By using intratracheal administration to induce tumour formation and to administer drugs, we limit the systemic effects that potentially could occur. We have also recently added intra nasal administration as a route of drug delivery as a further refinement of our protocols. Using oral gavage to induce recombination in the colon model reduces the risk of infection associated with intra-peritoneal injection whilst at the same time generates tumours in more than 95% of all animals. Where scientifically relevant, superficial tumours are used to complement the surgical model. We strive to always use the least invasive methods available to generate tumours or to add challenges or treatments. The tumour models chosen in this application reflect both the current clinical and in vitro studies ongoing in the laboratory and will be an invaluable complement to both. The methods outlined in the various protocols have been chosen to limit both animal numbers and suffering whilst at the same time provide robust scientific data. Invasive recovery procedures are kept to the minimum required for the experiment; analgesics and anaesthetics will be used where necessary.

Why can’t you use animals that are less sentient?

The mouse is the ideal model organism for our proposed work on tumour heterogeneity and therapy responses. While lower genetic models such as the fruit fly and yeast provide fundamental insights into chromatin regulation, they are not suitable for understanding tumour biology at this level, given the molecular heterogeneity, therapeutic responses and similarity with the human cancers demanded here. Mouse tumour biology closely models

many aspects of human cancers. Data from ‘co-clinical’ trials have shown that mouse models can accurately mirror data from human clinical trials and provide predictive data to improve clinical strategies. The use of mouse models also offers inbred lines with well-described differences, optimised standard protocols for husbandry, transgenic technologies, and a broad repertoire of tools for genetic manipulation and tumour tracking that are not currently possible through the use of clinical samples alone.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

At our establishment we have access to excellent veterinarians and animal technicians with clear procedures in place in the case of animal concerns, should these arise. We are constantly aiming to improve our experimental models to ensure that we are minimising any harms to the animals. All animals are routinely monitored for adverse health issues. To minimise the harms to the animals, analgesics and anaesthetics will be used where appropriate and we follow local rules and guidance on post-operative care and pain management. We are using score sheets for animals subjected to brain tumours in order to achieve consistent monitoring between the experimental animals and to have clear harms criteria for the animal staff, enabling any mice that approach the humane endpoint to be humanely culled as early as possible. We thereby also increase the reproducibility of the experiment, which in turn reduces the number of animals needed to reach solid scientific conclusions. By performing a scruff-test on animals with lung tumours, tumour burden is detected much earlier than by weight loss, which results in termination of the experiment before clinical signs can be observed in the resting animal.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We are following the "LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery".

In order to ensure that the experiments are performed in the most refined and reproducible way, we take guidance from the following publications:

Smith, A. J., Clutton, R. E., Lilley, E., Hansen, K., & Brattelid, T. (2018). PREPARE: guidelines for planning animal research and testing. Laboratory animals, 52(2), 135–141. https://doi.org/10.1177/0023677217724823

Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al. (2020) The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol 18(7):e3000410. https://doi.org/10.1371/journal.pbio.3000410

Members of the Joint Working Group on Refinement: D. B. Morton (Chairman), M. Jennings (Secretary), A. Buckwell, R. Ewbank, C. Godfrey, B. Holgate, I. Inglis, R. James, C. Page, I. Sharman,
R. Verschoyle, L.Westall & A. B. Wilson (2001) Refining procedures for the administration of substances https://doi.org/10.1258/0023677011911345

P Workman, EO Aboagye, F Balkwill, A Balmain, G Bruder, DJ Chaplin, JA Double, J Everitt, DAH Farningham, MJ Glennie, LR Kelland, V Robinson, IJ Stratford, GM Tozer, S Watson, SR Wedge, SA Eccles, An ad hoc committee of the National Cancer Research

InstituteObservers: V Navaratnam17 and S Ryder, (2010) Guidelines for the welfare and use of animals in cancer research https://doi.org/10.1038/sj.bjc.6605642

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

At the establishment where we are located, we regularly receive information from NC3Rs and NORECOPA regarding advances in the 3Rs. New advances are implemented where scientifically possible. When becoming aware of new advances in the 3Rs, we also share these with collaborators outside of our institute.

184. Humanised Mouse Models for Public Health Research

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Humanised mice, Cancer, Immune safety, Biological medicines, Pathogens

Animal types	Life stages
Mice	juvenile, adult, neonate, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The main objective of this licence is to employ mice engrafted with human cells or tissues (humanised mouse models) for evaluating the potency and safety of novel biological medicines including biotherapeutics, vaccines and advanced therapies. These studies will be conducted in support of public health and are designed to reduce health risks and maximised patient benefits for novel biological medicines. Furthermore, insight gained in these studies will inform general regulatory considerations associated with similar novel biological medicines.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Animal models and in vitro assays are more often than not poorly predictive for clinical responses of biological medicines including biotherapeutics and advanced therapies. Humanised mouse models represent in many cases the optimal experimental setting to explore novel therapies. This includes the evaluation of their potency and safety but also the investigation of their mechanistic activities, which in turn enables further development of promising biotherapeutic candidates.

What outputs do you think you will see at the end of this project?

We will analyse the response to a range of biological medicines in humanised mouse models and establish if these models could be used as a predictive preclinical model for specific biological medicines. We will also perform research into the potency and safety of novel biological medicines. At the end of this project, there will be new information on the potency, safety and mechanisms of action for a number of novel biological medicines including cancer therapeutics and advanced therapies. This information will provide new insight and will assist in regulatory considerations regarding pre-clinical studies of similar novel biological medicines.

Who or what will benefit from these outputs, and how?

Short-term, science will benefit from this project through a greater understanding of the mechanisms of responses to new biological medicines in these humanised mouse models. During the course of this licence, validated and standardised assays using mice engrafted with human cells will be established and will lead to a reduction in the number of mice required to obtain predictive data. Long-term, people will benefit from this project through the use of proven safe and effective biological medicines. Short- and long-term, through the validation of predictive assays for preclinical evaluation of novel biological

medicines using mice engrafted with human cells, there will be replacement of non-human primates used for these kind of studies.

Specific benefits for the different objectives are as follows:

Objective 1. Immunotoxicity Studies

Benefits of the immunotoxicology studies will be the establishment of suitable protocols for evaluating potential side effects of biological medicines. These studies will also help to understand the mechanism of action of these therapies and develop potential strategies to reduce potential side effects on the immune system. Establishing relevant models, will benefit patients by ensuring that novel biotherapeutics and cell therapies are not at risk of causing adverse events and therefore are safe to be used in human. These outputs would be used by the wider scientific community in order to further develop/refine humanised mouse models for predictive immunotoxicological analysis of novel biotherapeutics. These benefits could be realised within the duration of this licence. A long-term benefit which may not be realised in the life-time of this licence would be to inform the scientific community that these less sentient animal models with relevant human immune system should be used instead of non-human primates in immunotoxicology studies.

Objective 2. Immunogenicity Studies

Benefits of the immunogenicity studies will be the establishment of suitable protocols for evaluating the potential immune response against a biological medicines that could decrease their efficacy. These protocols may then also be used to evaluate immunomodulation of this immune response. The data generated will indicate whether these protocols and the humanised mouse model are able to mimic and assess/predict responses to cell therapies and recombinant proteins with regard to immunogenicity and efficacy. Data will be published in peer reviewed journals and presented nationally and internationally. These outputs can be used by scientists and clinicians working in cell therapy/transplantation and biotherapeutic immunogenicity areas in academia and industry, and can also be used by regulatory bodies to help them make informed decisions on what assays to use and/or data to demand in licence applications. These benefits could be realised in the short-medium term (protocol development for immunogenicity and immunomodulation of cell-based therapies) within the duration of this licence.

Objective 3. Infection Studies

Benefits of the infection studies in the short term will be to define and standardise protocols for infection with human malaria parasites in humanised mice. Once assessed these protocols may then be used to investigate natural immune responses to human pathogens in a controlled experimental setting. The data generated by infection studies will be shared with the wider scientific community through publication in peer-reviewed journals, presentation at international and national conferences and dissemination, as appropriate, to the general public (for example via the Institute website). The benefits of the dissemination of the findings obtained could in the long-term be the harmonisation and standardisation of protocols for the generation of humanised mice and infection studies. This would lead to data that can be compared between laboratories. Another benefit of the infection studies is that they may replace in the medium to long term the use of non-human primates for the study of the human malaria parasites Plasmodium falciparum and Plasmodium vivax. Until the genetic engineering enabling the engraftment of human cells in mice was established, human restricted pathogens could only be studied in non-human primates (e.g. malaria, HIV). For Plasmodium vivax the need for the establishment of a small animal model is all the more important as the parasite life cycle stages associated with clinical disease cannot be studied in vitro and currently the only available model uses a closely related parasite Plasmodium cynomolgi that naturally infects macaques. Finally, a long-term benefit of the work done is the reduction of the burden of human disease in endemic populations gained through a better understanding of parasite biology and host interactions using a small animal model that better represents human infection and immunity.

Objective 4. Cancer Therapy Studies

The cancer therapy studies will establish the potency and safety of novel cancer biotherapeutics in comparison to existing biotherapeutics and will provide insight into their mechanisms of action.
Thereby, these studies have the potential to enable further modification of the novel biotherapeutics with the aim to make these novel therapies safer and more effective. Data will be published in peer review journals and presented nationally and internationally. The benefits of this study could be realised during the duration of this licence with scientists working in the cancer therapy field taking these outputs on board to further develop and optimise similar cancer biotherapeutics.

How will you look to maximise the outputs of this work?

We are working closely with developers of humanised mice to facilitate the development of new models with improved translatability to human response. Data will be published in peer review journals and presented nationally and internationally in scientific meetings. These outputs can then be used by scientists working in academia and industry and in regulatory bodies and help them to make informed decisions on what assays to use for or to demand in licence applications.

Species and numbers of animals expected to be used

- Mice: 3500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using immunodeficient mice which are genetically engineered to enable engraftment of human cells or tissues such as the liver, red blood cells, components of the immune system. Humanized mice allow us to address biological questions in a small animal model that can better reflect human-specific responses.

Juvenile mice are used for cord blood cells engraftment as this model requires around 18 weeks for efficient humanization. Adult mice are usually used for other human cells engraftment such as peripheral blood mononuclear cells (PBMC) that are white blood cells or red blood cells.

Typically, what will be done to an animal used in your project?

Mice will be engrafted with human cells or tissues such as human blood cells including human immune cells and human tumour cells. For this, human cells (cord blood cells, PBMC) are usually injected intravenously or intraperitoneally for red blood cells. Upon successful engraftment, mice will be treated with biological medicines such as for example immunotherapeutic antibodies, vaccines, cell therapies and other biologics. The route of administration (intravenously, intraperitoneal, intramuscular or subcutaneous) will be dependent of the type of biological medicine tested in order to mimic clinical administration of similar biologicals. Successful engraftment can be established within days or may take up to 16 weeks in case of engraftment of mice with human stem cells. Hence typical experiments can last around 4 weeks or up to 24 weeks depending on type of engraftment.

In order to investigate the mechanisms of action of the studied medicines, mice may undergo additional procedures requiring the injection of reporter molecules, reporter cells or other reagents. In addition, in some cases, mice may be infected with pathogens such as malaria parasites to evaluate the efficacy of the investigated therapy. Similarly, mice may be engrafted with tumours to evaluate the efficacy of the therapeutic intervention by measuring tumour growth.

What are the expected impacts and/or adverse effects for the animals during your project?

Generation of humanised mice:

Mice will be engrafted with human cells or tissues. Humanised mice engrafted with PBMC might develop mild graft-versus host disease (approximately 10-20% of animals) from 15-day post PBMC infection. Signs are piloerection, hunched posture, weight loss, reduced food and water intake, abnormal respiration. Some strains (e.g. NOG mice) are severely immunocompromised and in case they are not properly housed may suffer from becoming infected with pathogens that wild-type mice can clear naturally. If housed appropriately in a barrier environment, the likelihood is very low (<1%).

Some immunocompromised strains of mice may show unexpected phenotypes e.g. NSG may display a progressive tarsal lesion. This lesion starts as an abnormal gait or swelling of one or both heels which could progress to skin ulceration. Calcaneal tendon injury related to the amount of collagen in the tendon is considered the primary cause of the lesion. Restraint frequency and method of handling are considered the most important disease determinants, resulting in both clinical disease and histopathological changes to the calcaneal tendon. Handling will be minimised and a refined restraint method avoiding pressure at the hind end will be used, along with supportive husbandry measures including softer bedding. Treatment with analgesics and/or anti-inflammatories may be provided under veterinary direction. A clinical scoring system will be used to track the progression and severity of lesions and to provide a humane endpoint.

Objective 1: Immunotoxicity studies:

Cytokine release syndrome may be observed following injection of some therapeutic products. This is characterised by high levels of cytokines released by immune cells. If it happens it is expected to occur early after injection within 24h and no longer than 72h. Signs are piloerection, shivering and/or hunched posture.

Objective 2: Immunogenicity

Cytokine release syndrome may be observed following injection of some therapeutic products. This is characterised by high levels of cytokines released by immune cells. If it happens it is expected to occur early after injection within 24h and not longer than 72h. Signs are piloerection, shivering and/or hunched posture.

Objective 3: Infection studies:

Infection with pathogens:

It is not expected that mice will experience any adverse effects from infection with plasmodium spp. However, they will be monitored especially when infection is undertaken with other experimental procedures that may cause adverse effects e.g. injection of red blood cells where approximately 2% of cases, animals may develop symptoms of shock following repeated intraperitoneal injection. Any animal showing respiratory distress e.g. gasping immediately following the administration step will be killed by a Schedule 1 method. Mice may also experience adverse events with treatment of immune modulatory compounds and the dosage of this is carefully administered to minimise this risk

Objective 4: Cancer studies:

Possible adverse effects of experiments involving the growth of tumours in mice include weight loss, weight gain, tumours impeding locomotion and tumour ulceration. Furthermore, mice with tumours may show signs of general ill health such as inappetence, piloerection and hunched posture, inactivity or diarrhoea. However, the likelihood of any of these adverse effects to occur is very low since animals will be culled when tumours reach a specific size and before tumour burden affects the general well- being of the mice. Any adverse effects of tumour growth are very unlikely to be transient and are expected to become more severe over time when the tumour growth further.

In some cases, adoptive transfer of xenografts such as human peripheral blood mononuclear cells (PBMC) can lead to graft-versus-host disease in mice. The likelihood of mice developing graft-versus- host disease is low since animals will be culled at specific time points before graft-versus-host disease is known to develop. However, should graft-versus-host disease manifest in mice, the adverse effects are not transient and will become more severe over time if mice are not killed.

Immunodeficient mouse strains may be used which are more susceptible to infections. However, immunodeficient mice are kept in a bio-exclusive environment such as individually ventilated caging or isolators and rarely experience infections. Should infection occur immunodeficient mice are unlikely to clear the infection irrespective of the pathogen.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The licence contains one protocol (protocol 6) classified as mild and five classified as moderate (protocols 1-5).

Under protocol 1 (human immune system engraftment), approximately 50% of mice may experience moderate severity.

Under protocols 2 (immunotoxicity studies) and 3 (immunogenicity studies), the severity will depend on the type of biotherapeutics and cell therapies tested. Some mice (approximately 50%) may reach moderate severity level under each of these protocols.

Under protocol 4 (infection studies) the expected severity will depend on the optional procedures used and study duration. Mice undergoing optional procedures such as irradiation, immune modulation and/or red blood cell engraftment may experience moderate severity. From experience, most animals will reach moderate severity levels under this protocol (approximately 20%) with the rest of animals experiencing mild severity levels.

Under protocol 5 (cancer therapy studies), mice undergoing optional procedures such as irradiation, adoptive transfer and/or immune depletion may experience moderate severity. Depending on the tumour model chosen, mice may reach moderate severity levels when the tumour reaches a certain size. From experience, only a proportion of animals will reach moderate severity levels under this protocol (approximately 40%) with the rest of animals experiencing mild severity levels.

Under protocol 6 (Breeding and Maintenance of GA animals), mice are not expected to experience more than transient discomfort.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The main objective of this licence is to compare humanised mouse models with in vitro assays and/or animal models (such as mouse models and non-human primate models where available) for evaluating the potency and safety of biotherapeutics. Our aim is to identify which humanised mouse models offer clinically relevant predictive advantages over in vitro assays and other animal models and which don't offer any such advantages and therefore should not be used. We have a particular interest in informing the public and the regulatory bodies of the outcome of our studies and, thereby, influencing the regulatory process of novel biotherapeutics. The benchmarks for vaccine and therapeutic product evaluations in humanised mice need to be set. While this process is ongoing there will be a requirement for using animals. Our study should identify for which aspects in vitro tests represent suitable alternatives. However, in some cases in vitro assays are unlikely to be suitable for thorough assessment of therapeutic intervention strategies. For the evaluation of immune responses to biotherapeutics, the activation of immune cells is often complex and requires the correct cell types to be spatially and temporally arranged within a suitable microenvironment as seen for example for vaccinations or for immunogenicity induction. As a result, it is often not possible to reproduce this in vitro. In vitro tests could form an alternative in the future but may not always be suitable for thorough assessment of therapeutic intervention strategies.

It is important that the assays reflect the human body as closely as possible. A typical example of wrong prediction of toxicity happened in 2006 during the preclinical study of the superagonist TGN1412. Both preclinical safety testings in vitro and in macaques failed to predict an adverse response to TGN1412 at clinical trial. Therefore, better assays that are equally suitable for a wide range of biotherapeutics could be instrumental in harmonising the immunotoxicological characterisation of novel biotherapeutics. The humanised mouse models have the potential to revolutionize immunotoxicology by making other animal models less important (e.g reduction of the use of Non-Human Primate) or even obsolete.

In addition, for a number of human infectious diseases the immune correlates are not known (e.g. malaria) and therefore, currently no suitable in vitro tests to determine protective efficacy can be used. In addition, some pathogens have complex life-cycles including several hosts or several tissues within a host; it is not currently possible to determine the effect of several life-cycle stages in vitro. As a result, it is often not possible to reproduce this in vitro.

Which non-animal alternatives did you consider for use in this project?

We are exploring alternative to the humanised mouse models for different study areas Immunotoxicity: in vitro cytokine release assays; published data from non-human primate studies Cancer Therapy: in vitro assays; conventional mouse models (separate PPL) Why were they not suitable?

The predictive index of in vitro assays is limited when studing off target side effect of immunotherapeutics (immunotoxicity, cancer therapy).

Immunogenicty: no tests available for predicting immunogenicity. in vitro and in silico test exist to assess immunogenicity

Infection/Vaccination (malaria): no alternative in vitro tests available; no alternative animal model available

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers are based on experience with the previous licence. Genetically modified animals produced under protocol 6 are transferred to protocols 1-5 for continued use. Similarly, animals engrafted with human cells may be transferred to protocol 2-5 for continued use. A further 1,000 animals are used under protocols 2-5 without prior engraftment under protocol 1 or breeding under protocol 6 bringing the total number of animals used under this licence to 3,500 animals.

Experiments will be discussed with biostatisticians to ensure that meaningful data will be obtained from each experiment with a minimum number of animals used.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Steps will be taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. operator is blind) following the ARRIVE guidelines. To reduce potential variability, the same source of hematopoietic stem cells will also be used for engraftment whenever possible.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All new studies or areas of work will be first subjected to pilot studies to refine procedures and experimental groups.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The main aim of this study to establish validated and standardised humanised mouse models for the investigation of therapeutic intervention strategies and to use these models to evaluate the safety and potency of novel therapeutics medicines. For understanding the potency and safety of biological medicines, the model must include an intact human immune system and suitable tools such as antibodies, recombinant factors and suitable tumour cell lines have to be available for enabling the mechanistic investigation of the model.

Immunocompromised mice with deficient or non-functional immune cells are used to generate humanised mice because their immunity is compromised making them unable to attack foreign cells.

These mice are therefore the best recipients for engraftment for human cells or tissues. Mice will be housed in specific filter cages or isolators to protect them from potential infection.

Mice may be irradiated to enable better engraftment with human cells. Irradiation dose will be kept to the minimum to avoid adverse effects. Similarly, mice may be treated with substances such as for example clodronate liposomes to enable better engraftment. Volume used for injection will be kept to the minimum and single use needle will be used. In most cases, mice will receive only a single injection of human cells. When repeated injection of cells is needed such as in the case of red blood cells, we will reduce the frequency and volume whenever possible (e.g, every other day instead of daily). Blood sampling will be used to monitor engraftment levels. Micro-sampling may be performed using capillaries for repeated sampling of the same mice from the tail vein.

Regarding the tumour models, the project will use human xenograft tumour models using either established human tumour cell lines or primary human tumour cells. The mice will be injected with immunotherapeutic medicines including cellular therapies and may also undergo whole-body imaging in specific experiments. Luciferase-expressing tumour cells may be used for whole-body imaging when available to allow for better monitoring of tumour growth especially concerning internal tumours. The use of luciferase-expressing tumour cells will allow to define more accurate humane endpoints for individual mice with internal tumours.

Anaesthesia of mice will be performed with isoflurane whenever possible. From our experience, mice undergoing anaesthesia using isoflurane do not experience any adverse effects. Only on rare occasions when usage of isoflurane is not advisable will injectable anaesthetics be used.

Why can’t you use animals that are less sentient?

Engraftment of human cells has been validated in these strains of mice. We are using animals at different stages of life depending on the type of cells used for humanisation (adult for PBMC; juvenile for stem cells).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our biological service division (BSD) has a very good track record in minimising harms to animals. Refinement of procedures implemented during the previous licence include:

- single use needles - using a new needle for every animal that is injected

- increased observation frequency after specific procedures (e.g. surgery, irradiation and adoptive transfer, injection of immunostimulatory reagents)

- supportive husbandry measures e.g. mash diet and additional warmth when anaesthesia is used or upon irradiation.

- non-aversive animal handling techniques

- various enrichments in the cages

The scientists, the NVS and the BSD staff working on a project have pre- and post-meetings for experiments. During these meetings possible refinements are discussed and jointly agreed.

In addition, experimental endpoints will be adjusted to deliver the minimal average tumour size that allows to achieve the scientific objective and the minimum irradiation dose that allows for good engraftment. Also, endpoints will be chosen to avoid mice showing signs of graft versus host Disease (GvHD).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We comply with the best practice guidelines such as the European Federation of Pharmaceutical Industries Associations (EFPIA) and the European Centre for the Validation of Alternative Methods (ECVAM) for administration of substances and blood sampling (Diehl et al., Appl. Toxicol.21, 15-23, 2001).

Regarding the tumour models, we will follow the Guidelines for the Welfare and Use of Animals in Cancer Research (Workman et al., 2010 British Journal of Cancer 102: 1555-1577).

Maximum blood withdrawal volumes and dose volumes for specific routes of administration will be adhered to as listed in the action plan. In addition, expert input from NVS and Named Animal Care & Welfare Officer (NACWO) will be thought for new treatment regimens.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We stay inform on new development in the field by attending conferences and webinars on this subject and by screening the literature.

Scientists, the Named Veterinary Surgeon (NVS) and the staff members performing the experiments in the biological service division (BSD) hold pre- and post-meetings for experiments. During these meetings all aspects of the experiments including the 3Rs are considered and discussed and are documented in the meeting notes. Refinements are jointly agreed on and implemented for future experiments following the same procedures.

185. Development, Validation and Deployment of Imaging and Related Technologies.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

in vivo imaging, body scanning, engineering, physiological monitoring and maintenance, motion control

Animal types	Life stages
Rats	neonate, juvenile, adult
Mice	neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This licence will be used to improve the technologies used for, and associated with, body scanning in small animals such that both the Refinements and Reduction aspects of the 3Rs are concurrently enhanced. The technology developments will be conceived, validated and developed to 'product quality' such that they can easily be deployed at establishments that image small animals.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Body scanning is established as a leading tool in the diagnosis, management and treatment of disease as it allows the doctor to examine the structure and function of organs repeatedly and without the need for any invasive procedures. Different scanners provide different information ranging from simple examinations of, for example, organ or tumour sizes, through to the real-time examination of molecular processes including metabolism. It is increasingly common in the clinic to use a range of imaging modalities in order to characterise disease effectively. Indeed multi-modal scanner technologies such as PET-MR and MR-guided radiotherapy are increasingly available as commercial products. Because of the large scale of market for these scanners and the stringent safety requirement of the clinic, the scanners are very well developed, can operate as as a 'push-button' technology, and are are located in all manner of clinical centres ranging from major research hospitals to the local GP surgery. Preclinical imaging scanners, those which are used to study models of disease in small animals, are much less common and much less well developed, typically needing highly expert imaging users to operate.
There is an increased drive in the preclinical imaging market to deliver imaging systems to biological application users, rather than just to the imaging scientist, and this requires that the systems deliver high quality data for the non-expert user. Engineering solutions aim to bridge-the-gap, but these frequently compromise imaging performance in order to deliver a greater adoption of the technology. In some cases, for exampl, those involving desensitisation to cardiac and respiratory motions, this compromise leads to prolonged scan times as data are often ‘oversampled’ (measured more than once) with computer methods sorting data to provide high fidelity images. Whilst this approach manages short term motions such as heartbeats and breathing very successfully, it renders the imaging process sensitive to longer term processes such as bladder filling and changes in body tone. It also prolongs the minimum necessary duration of anaesthesia as usually used for immobilisation of the animal during the scan. Faster imaging using appropriate control systems can, therefore, reduce both image corruptions and anaesthetic durations so improving data quality and animal welfare concurrently. Previous developments have shown that this can be achieved in a manner that is appropriate for use by the applications user without day-to-day attendance by the imaging scientist so increasing the availability and uptake of these minimally invasive technologies. Such developments will continue with this work.

Furthermore, there is no operational framework for vendors to make commercial imaging systems arbitrarily compatible with one another, so there are no generally available means for transferring animals arbitrarily between different imaging systems. This hinders the development and uptake of multi-modal imaging; a combined technique which involves use of more than one imaging technique and which can provide exquisite detail of multiple biological phenomena simultaneously. This work will continue a program of development of an operational and technological framework that will resolve the issues of inter-scanner compatibility and enable the simple and rapid deployment of such multi-modal imaging techniques.

Operation of a program of Research and Development for the improvement of the image acquisition process will provide direct benefits to image quality, animal welfare and integration of imaging into the scientific discovery process. New and better scan modes can also examine aspects of biology that were not previously measurable and can improve both the sensitivity and specificity of existing scans. Faster scanning and the use of new and better physiological monitoring and maintenance apparatus will provide direct

improvements to animal welfare. Better animal handling apparatus will enable animals to be transferred between, otherwise incompatible, scanners in order to make multi-parametric measurements that, again, improve the sensitivity and specificity of imaging and improve the translational potential of in vivo biology research.

Once methods are developed and validated, they can be distributed to the wider scientific community and so provide opportunities for accelerating the clinical uptake of translational developments.

What outputs do you think you will see at the end of this project?

New and better imaging scan methods, animal monitoring systems and other support equipment that are all compatible with a range of imaging scanners will be available. Technical developments will be described in the scientific literature, through public presentation and via web-distribution. Many of the technologies being developed are generally applicable, so can be applied independently of the physiology or disease to be studied. The distribution and uptake of these techniques will improve the quality of imaging works performed by a diverse user community which studies a wide range of physiological and diseased states, so providing a range of opportunities for clinical translation of both imaging methodology and biology.

Who or what will benefit from these outputs, and how?

The local imaging user groups will be the early adopters of the technologies as they are developed and so be the first to benefit. During this distribution the methods will reach maturity, allowing public presentation and publication, and the methods will become available for other groups that are collaborating with the local imaging group. At around this point detailed technical descriptions will be made publicly available for any other groups to replicate. Where possible, commercial suppliers will be offered the technologies in order to maximise opportunities for distribution. This will provide direct benefits to the animals as we expect to demonstrate both reduced imaging/anaesthetic durations and improved physiological maintenance.

How will you look to maximise the outputs of this work?

Presentation at imaging, biomedical and animal welfare conferences and other meetings.
Publication in imaging, biomedical and animal welfare journals.
Development of collaboration with other sites requiring and/or developing such technologies. Distribution of methods and descriptions via web-based repositories.
Species and numbers of animals expected to be used

- Mice: 1000
- Rats: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mouse and rat are the rodent species most examined with whole body imaging. Neonatal animals will be used in the development of apparatus and scan modes that will become available for use in the examination of the rapidly developing brain and body.

Juvenile and adult animals represent the majority of animals to be used, and will be studied in the develpment of apparatus, scan modes and workflows that will be appropriate for use in a wide range of disease areas and organ systems.

Typically, what will be done to an animal used in your project?

Typically, an adult animal will be anesthetised, its tail vein will be cannulated with small diameter needle, and it will be placed in an imaging cradle featuring integrated head immobilisation, temperature control and vital sign monitoring for up to 2 hours, and then recovered. Imaging tracer will be delivered via the tail vein during imaging. This will be repeated, twice per week for up to one month. The animal will then be killed and tissue taken for post mortem analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals will experience the impact of general anaesthesia and tail vein cannulation. Good quality control of temperature and depth of anaesthesia throughout the procedure, hydration of the animal and the use of small diameter cannulae will minimise the impacts whose effects are expected to be resolved within a day of imaging.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice. Non-recovery 50%. Moderate 50%.

Rat. Non-recovery 50%. Moderate 50%.

Neonatal and juvenile animals are anticipated to account for <20% of animals used.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The development of improved methods and apparatus for imaging of small animals has a fundamental requirement, at least at the validation stage when methods are to be tested in a real-use manner, to use living animals.

Which non-animal alternatives did you consider for use in this project?
New concept developments are initially made to work 'on-the-bench' using test samples such as gels, and electronic control systems. These mimic the operation of, for example, the scanner, the beating heart and the breathing lung.

Once successfully demonstrated 'on-the-bench' operations are repeated 'on-the-scanner', again with test samples and appropriate control systems. An iterative optimisation of the techniques is often then employed until the new development is successfully demonstrated 'on-the-scanner'.

Only when 'on-the-scanner' use of the new development has been successful will operations proceed in vivo. Data will then be collected, analysed and reported to validate the new development and to support its wider deployment.

Why were they not suitable?

Early stage method development and testing allows significant progress to be made without any need for an animal. However, this cannot always mimic the in vivo situation in which a range of technical

obstacles (including cardiac, respiratory, peristaltic and bladder filling motions; respiratory depression; and compromised thermoregulation) are simultaneously present.

Only when methods have been developed to the point at which they will operate successfully in vivo,
and when in vivo data are required to support the validation of the development will operations switch to the use of live animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are based on prior experience of imaging technology development. A new technical development will typically require 10-20 animals for early stage transfer from development stage to validation, 10-20 animals for the validation and generation of data for peer review and a further 20 for long term evaluation of the stability of the technique. This may need to be repeated over a range of life stages. Early stage developments will typically use animals once only, but studies performed in order to validate use of the development are likely to require repeated imaging.

Other investigations into the sensitivity and specificity of existing imaging techniques will use similar numbers; 10-20 for definition of image presentation and 10-20 for systematic examination of this.

These studies will provide the data that declare the need for a technical development or otherwise. The total numbers are based upon 20 studies/developments in the mouse and 2 in rat.
What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In vivo testing is only used once methods have been shown to work to specification using a variety of test systems including test samples and with the image acquisition controlled by synthetic test signals such as simulated respiration and ECG traces.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Animals will be used in terminal experiments wherever possible with recovery only being used where some long-term assessment of a biological development of inter-scan stability is required.

The project format is entirely compatible with adaptive study design in which study stops once robust performance (or otherwise) is established.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice that are not genetically altered, or that are genetically altered with no harmful phenotype at the time of use, will form the majority of animals entering this work, as most imaging technology developments can take place without the need for disease. Some non-genetically altered rats will also be used.

A range of imaging techniques will be used and developed in order to deliver the maximally refined experimental apparatus and imaging experiment. These scans will offer a combination of faster scanning with a commensurately reduced duration of anaesthesia, improved data quality, and the delivery of methods that can operate robustly in the hands of trained, rather than technically expert, operators, so promoting the uptake of the imaging technologies in the general scientific community.

Once the validation stage is complete the technology will be made available to others with authority to image animals.

Why can’t you use animals that are less sentient?

The choice of species is governed by the unmet needs in preclinical imaging and biological research. Most preclinical whole body imaging is performed in the mouse and rat; lower

species do not always present the biological features of interest sufficiently well and higher species are not necessary.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The program of work is targeted at improving imaging and the animal experience during experiments; the deployment of faster imaging allows reductions in imaging time, and deployment of better animal maintenance apparatus maintains better animal physiology. When developments in technology are made appropriately then shorter anaesthetic times can be used on better maintained animals.
Furthermore, the methods will be developed to 'product standard' so enabling their use by non-expert imaging personnel. All experiments will be designed and performed in conjunction with our institute's veterinary and in vivo support teams so that welfare benefits can be maximised. A strict monitoring of animal behaviour and health will be performed so as to ensure that the new developments invoke no negative harms upon the animals, and these assessments will constitute part of the valiation process. The deployment of new and validated imaging methods will lower the entry barrier to imaging so providing opportunities for imaging to replace more invasive and sample-intensive measurement tools such as post mortem histology.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Workman et al, 2010 Br. J. Cancer. Guidelines for the welfare and use of animals in cancer research

Percie du Sert et al, 2020. PLOSBiology. https://doi.org/10.1371/journal.pbio.3000411. Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am an active participant in the institutional 3Rs programme and a member of its 3Rs review committee, and a participant in national 3Rs events.

Much of the work will be directly aimed such that improvements to 3Rs practice result naturally from the imaging-related developments.

186. Neural Circuit Dynamics of Memory in Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Neuroscience, Memory, Neurodegeneration, Memory

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overarching aims of this project are to generate fundamental understanding of the mechanisms that enable experience (or episodic information) to be processed and stored in the brain and to understand how these mechanisms are disrupted by disease pathologies that cause memory loss. Additionally, we aim to show that manipulating the properties of brain circuits using drugs or non- invasive brain stimulation can rectify malfunction and thus restore memory in pathological states.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
Developing and validating treatments that can improve or recover memory in people depends on having a fundamental understanding of how brain circuits involved in this process should work correctly, and how they are disrupted by disease. The purpose of our work is to identify pathways to new clinical interventions that can ameliorate dysfunction in neural circuits that cause memory loss in people suffering from neurological diseases, in particular dementia.

What outputs do you think you will see at the end of this project?

By the end of this project, we will have generated substantial new understanding of how neural circuits process episodic memories and how these circuits malfunction in neurological diseases that cause memory loss. The data outputs that will provide these insights will principally be measures of the structure and activity patterns of cells comprising neural circuits and behavioural measures that relate the structure and activity of these cells to episodic memory. Additionally, we will generate data on drug and brain stimulation interventions that can modify neural circuit properties. The datasets we will generate will contain complex multimodal information, and thus, where there are not conflicts of interest, we will freely share our data to allow other researchers to validate our findings and make discoveries from the work. The findings of our research will mainly be communicated via peer-reviewed publications in academic journals, presentations to other researchers at conferences, and dissemination to the pharmaceutical industry through our current and new collaborations. Where appropriate, we will also communicate our findings to the public through organised outreach events.
We also have a track record of communicating our published research to the public through press releases in digital and print media.

Who or what will benefit from these outputs, and how?

The main short-term beneficiaries will be scientists interested in memory and memory disorders, and pharmaceutical and other private sector companies interested in developing therapies for cognitive disorders. In addition to knowledge outputs, these stakeholders will benefit from the skills attained by researchers trained under this project. As we expect the project to generate knowledge that informs the development of novel cognition-enhancing therapies, we hope that people suffering from dementia and related cognitive disorders will benefit within a reasonable timeframe for therapy development – around 10-20 years. Additionally, we expect that advancing knowledge of neural mechanisms underlying memory will benefit a range of stakeholder groups, including the education sector and computer scientists developing artificial intelligence algorithms. We also hope that these groups will benefit within a medium-term timeframe of 5-10 years.

How will you look to maximise the outputs of this work?

To maximise the drug discovery potential of this project, we collaborate with the pharmaceutical industry and will explore opportunities for synergies with the pharmaceutical industry to access novel biologicals aimed at enhancing cognitive abilities or clearing dementia-related pathologies. Additionally, we aim to develop our collaborative links with private sector companies specialising in ultrasonic equipment that have interest in developing products for non-invasive brain stimulation. Alongside these partnerships, we collaborate with researchers using post-mortem tissue from human patients to characterise genetic signatures of dementia and develop novel diagnostic assays. By integrating our preclinical mechanistic studies with analyses of patient data we aim to

maximise the translatability of our research to increase its potential for improving people's lives.
Where our findings are not commercially sensitive, we will aim to disseminate them as widely as possible. We have a track record of publishing research in open access journals, and we will continue to make every effort to ensure that our research does not require a paid subscription to be viewed. To this end, we will disseminate our results using open access pre-print repositories such as biorxiv.org prior to publication in peer reviewed journals. Findings in this project that do not support our hypotheses (i.e. 'negative' results) will still provide important information about how the brain is working, and so we will disseminate these findings via the outlets described above.

Species and numbers of animals expected to be used

- Mice: 5000
- Rats: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The aims of this project cannot be met using non-mammalian models, such as in silico simulations, cultures of immortalised cells, or non-protected animal species. This is because the organisational,

functional, or behavioural complexity of these models limits their generalisability to humans, such that brain circuits that generate measurable cognitive abilities in people cannot be mapped to comparable circuits in these models. The aims of the project also cannot be achieved in human volunteers as current methods to measure brain structure or function in people do not deliver the necessary resolution to generate information about how individual brain cells and specific types of brain cell communicate cognitive information. We will use mice and rats because the anatomy and physiology of rodent brains is comparable to humans such that cognitive processes like episodic memory can be attributed to equivalent brain regions, and because methods that generate information about cellular function that this project seeks to attain are well established in rodents.

Most experiments in this project will use mice. This is because mice have been used extensively as a model to study how cognition is generated through brain activity, and this project will build-on existing results and methodologies that are well validated in the literature, including in our published work.
Additionally, we aim to use several advanced genetic tools for studying brain function that have been developed in genetically altered mice, and genetically altered mouse models of dementia that are well validated in the literature, including by our own studies. Rats are more intelligent and have better memory ability than mice, and so small proportion of experiments may be performed in rats where they constitute the most refined model to complete an experiment.

Memory is a process that is differentially regulated across the life course. Experiments to investigate fundamental mechanisms of memory will mainly be performed in young adult

animals. Consistent with this approach, our studies of dementia will focus on prodromal (early stage) disease, when memory loss first emerges. However, dementia typically manifests late in life, and, as in people, dementia- causing pathologies develop progressively with age in genetically altered animal models.
Consequently, to contrast characteristics of prodromal dementia with more advanced clinical phases of the condition it will be necessary to study some older animals, beyond 15 months of age.

Typically, what will be done to an animal used in your project?

Many of our experiments will require cranial surgery, performed under general anaesthesia. In most cases, we will infuse genetic tools into the brain that enable specific cells to be visualised and/or their activity to be modulated. These tools are extremely powerful, since they allow specific groups of cells to be studied in a targeted manner, reducing the number of recordings needed to attain sample sizes of adequate statistical power, and because they enable experiments that can establish causal relationships between the function of specific cells and memory abilities. In these cases, surgeries will last around 60-90 minutes. We will make a hole in the skull and insert a fine needle (around the width of a human hair) into the brain. After infusing a small amount of substance into the brain (around 1 µl) we will reseal the skull and allow the animal to recover from anaesthesia. Typically, animals experience some postoperative pain that can be effectively managed using analgesic medication, under the guidance of a vet. Animals typically recover within a few days. They are housed under standard conditions for several weeks to allow time for the genetic tool to express, after which they are killed under deep anaesthesia so that their brain can be used for study.

In a smaller group of experiments we will also surgically implant optics or electrodes into the brain that allow us to measure and modulate the activity of specific groups of cells whist animals carry out simple behaviours, such as exploring an arena or maze. Such experiments are crucial to this project, as they enable us to directly relate brain function to memory abilities. A small device (weighing a few grams) is

implanted into the brain under general anaesthesia and secured. Postoperative pain can be effectively managed using analgesic medication (under veterinary guidance), and animals typically recover within a few days. Recordings will commence once animals have recovered from surgery (determined via wellbeing assessment), often in tandem with behavioural assessment. In most cases, all surgical procedures will take place under a single bout of general anaesthesia, lasting around 90-150 minutes. However, in some cases, the experimental design may be refined by performing an intracranial infusion in one surgery and implant in another, such as where it is necessary to express a cellular marker at a single age point in all animals in a study, but to measure the marker different age points (e.g. different pathological stages in a dementia model). This approach spreads the time that animals are under general anaesthesia across surgeries (e.g. 60 min plus 90 min) and can thus decrease the amount of time that older animals spend under general anaesthesia. Follow up surgery will not occur until animals have recovered from the first.

For electrode recordings, the implanted device is connected to a recording apparatus via a lightweight wire; for optical recordings, the implant is fixed underneath a microscope. These procedures do cause animals some initial stress, which can be controlled by allowing them to gradually habituate to the recording apparatus over several days. The recording apparatus is tailored to ensure that animals are comfortable and can move as

normally as possible, and, in our experience, animals quickly get used to the apparatus and readily engage in natural behaviours such as grooming, feeding, and sleeping. Indeed, because animal behaviour is a key measure of cognitive ability, ensuring that animals feel comfortable enough to behave naturally is key to the success of our experiments. For most experiments, animal's behaviour will be measured for a few days, but for some more complex tasks or experiments that assess long-term memory, it will be necessary to measure an animal's behaviour for several weeks. At the end of experiments, animals will be killed under deep anaesthesia and the brain removed to enable further examination of anatomical, functional, or genetic changes.

What are the expected impacts and/or adverse effects for the animals during your project?

Pain: animals experience some postoperative pain following cranial surgeries. This typically lasts for a few days and can be managed using appropriate analgesic medication, under veterinary guidance.

Weight loss: general anaesthesia can cause short-term loss of appetite, resulting in weight loss immediately after surgery. This can be assessed by monitoring animals' body weight postoperatively and providing supplementary appetitive foods such as high calorie diet gel and mashed chow.

Stress: connecting an implanted recording device to a wire or head-fixation clamp causes animals some initial stress. This can be controlled by allowing animals to gradually habituate to the recording apparatus across several days. Because we are interested in linking neurophysiological measurements to animal behaviour, it is essential that animals feel comfortable enough to behave naturally whist connected to the recording device.

Cognitive impairment: animals that have been genetically altered to model dementia exhibit symptoms of memory loss. These symptoms, however, do not typically manifest as obvious behavioural changes until late stages of disease, and thus require specialised tests to be observed. Most of our experiments will focus on prodromal dementia, and so few animals will reach advanced disease stages in which behavioural abnormalities may occur.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

A large proportion of animals (around 50%) will be genetically altered animals that are bred to sustain colonies (for the purpose of creating experimental animals) and to provide tissues for anatomical or genetic analyses. These animals will suffer mildly.

The remaining 50% of animals will experience moderate suffering caused by surgical and/or behavioural procedures. Of this class of animals:

- Approximately 40% will only experience recovery intracranial infusion surgery.

- Approximately 10% will experience recovery intracranial infusion surgery in conjunction with optical or electrical recording under non-recovery anaesthesia.

- Approximately 20% will experience recovery implant surgery followed by neurophysiological recording and behavioural analysis.

- Approximately 30% will experience recovery intracranial infusion and implant surgery followed by neurophysiological recording and behavioural analysis.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The brains of non-human mammals are a crucial model for analysis of human cognitive systems because many features of brain anatomy and function are conserved across mammalian species. Rodents are an ideal mammalian model for this study because they can be readily combined with physiological recordings and genetic manipulations that enable expression of disease pathologies and/or genetic tools that can be used to test mechanistic causality. These genetic manipulations cannot be so readily achieved in other mammalian models, such as non-human primates.

Which non-animal alternatives did you consider for use in this project?

- In silico models

- In vitro cell cultures

- Electrophysiology in humans

- Imaging in humans

Why were they not suitable?

In silico models: we actively collaborate with computational neuroscientists to build simulations from our experimental findings and we will continue to use computer models where possible, since this approach maximises the outputs from our data and aids the refinement of our experimental design. However, current computer models are simplified representations of neural circuits that need to be constructed using known parameters that can only be obtained from experimental measurements.

In vitro cell cultures: brain cells can be grown in culture and electrophysiology and/or imaging used to measure their properties. However, cells in culture form connections that bear little resemblance to neural circuits in the mammalian brain. Additionally, cell cultures are made from stem cells or juvenile tissues and therefore typically display immature

characteristics. This project is principally concerned with studying mature neural circuits, since memory impairment and dementia typically manifest in adulthood.

Electrophysiology in humans: as we are interested in understanding how neural mechanisms of memory become disrupted in disease, human patients are the best experimental system. However, non-invasive electrophysiological techniques, such as electroencephalography (EEG), have limited anatomical resolution (on the order of cm3) that precludes the study of individual cells and their connections (µm3) that constitute the focus of this project. Cellular recordings can be made using implanted electrodes in some patients with epilepsy and Parkinson's disease. However, opportunities to perform these recordings are very rare and confounded by the underlying neurological conditions in these people. Such recordings are not typically performed in people with dementia. These recordings also cannot deliver the synapse-level data that will be generated in this project. Imaging in humans: human brain imaging techniques such as magnetic resonance imaging (MRI) and functional MRI (fMRI) can generate useful clinical information on brain structure and areas that are activated during cognitive tasks. However, like EEG, these techniques lack the anatomical resolution (mm3) to generate insights into synaptic and cellular mechanisms of memory or disease pathogenesis. fMRI also uses cerebral blood flow as a proxy measure of neuronal activity and can therefore be subject to interpretative errors, particularly when studying dementia-causing illnesses like Alzheimer's disease that are characterised by vascular dysfunction.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The specified animal numbers are based on:

- Extensive experience of carrying out the types of experiment detailed in this project, which has informed the project design.

- Use of statistical models to estimate animal numbers required to appropriately power analyses of the proposed experiments.

- Knowledge of the proposed animal models and usage of these models by similar studies in the literature.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

- Our experimental design is informed by use of validated statistical software, including GPower 3.1 for power calculations (Faul et al. 2009. Behav Res Methods. 39:175-91) and the NC3Rs Experimental Design Assistant (Percie du Sert et al. 2017. Nat Methods. 14:1024-5).

- We use power calculations (typically based on achieving 80% power at an alpha level of 0.05) to generate a priori estimates of sample sizes needed to power statistical analyses for each experiment. Our estimates of experimental effect size are based on our prior work using similar techniques, on published literature where this is more appropriate, or (in rare cases where neither of these is available) on the smallest biological effect that could be deemed meaningful.

- Where appropriate, we use a longitudinal study design. This reduces inter-animal variability (since intra-animal controls are used) and enhances statistical power, thus reducing the number of experimental subjects needed.

- Where it is appropriate to employ a nested experimental design in which multiple samples (e.g. cells) are measured in a single animal, our typical statistical approach is a hierarchical mixed model analysis. This method attributes variance at each analytical level (e.g. cell, animal, cage) and we ensure that each level is suitably replicated in the dataset (typically a minimum of 5 data points at each level).

- We maintain collaborations with academics that have established experience in designing tasks to assess episodic memory in freely behaving and in head-fixed animals. These colleagues have been and will continue to be consulted to optimise the design of specific experiments to reduce the number of animals needed to achieve sufficient statistical power.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Use of genetically altered animals

Three general classes of genetically altered (GA) animals will be used in this project:

- Reporter lines: animals harbouring exogenous genes that express fluorescent markers, activity probes or functional actuators in specific cell types.

- Cre-driver lines: animals that express Cre recombinase in specific cell types. Cre recombinase is an enzyme that can edit DNA sequences that block gene transcription. It can therefore be used to express genetic tools in specific cell types by crossing with reporter lines or following viral transduction.

- Dementia models: animals harbouring mutations in genes that cause dementia or increase dementia risk in people.

Using GA reporter or Cre-driver animals in concert with genetic tools allows us to optimise the economy of our experimental recordings. For example, it is not possible to visualise specific cell types (e.g. excitatory vs. inhibitory neurons) in wild-type animals, meaning that surplus recordings must be made to sample a subpopulation of interest. GA reporter/Cre-driver animals enable observable non- toxic markers to be expressed in specific cell types, which allows them to be recorded in a targeted manner. This prevents cells outside of the target population from being recorded in error, thus reducing the number of recordings and therefore animals needed per experiment.

Efficient breeding

We will use efficient breeding strategies, based on genetics, to minimise numbers of experimental animals that are bred. For example:

- Reporter and Cre-driver lines will be preferentially bred to homozygosity (transgenic DNA inherited from both parents) to reduce the generation of non-experimental animals.

- Models of dementia will be preferentially bred as heterozygote pairs (transgenic DNA inherited from just one parent) , which will provide both wild-type and heterozygous control animals for experiments. This is statistically the most effective study design and reduces the need to breed wild- type control animals.

Reduction of variability

- Where appropriate, we will use a longitudinal study design. This reduces the impact of inter- animal variability (since intra-animal controls are used) and enhances statistical power, thus reducing the number of experimental subjects needed.

- Animals will be exposed to consistent housing and experimental conditions by controlling variables such as noise, light levels, diet, and home cage enrichment.

- We will minimise the age range of animals used in a particular experiment.

- We will use food or water rationing to motivate animals to perform behavioural tasks. This procedure reduces inter-animal variability in task performance and ensures sufficient behavioural sampling in individual animals to power statistical analyses.

- We will use non-Schedule 1 methods of killing (e.g. decapitation under anaesthesia; perfusion fixation under anaesthesia) where appropriate to enhance the viability of ex vivo tissues. By

maximising the tissue quality we will maximise the number and successful experiments and reduce inter-experiment variability.

In silico hypothesis testing

- We maintain active collaborations with computational scientists to develop in silico models based on our experimental findings. These models can be used to make a priori predictions to refine our experimental design.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animal models

- Mice will be the primary model species in this project. Mice have been used extensively as a model to study how cognition is generated through brain activity, and this project will build-on existing results and methodologies that are well validated in the literature, including our own published work.

- We will use several advanced genetic tools developed in genetically modified mice that allow experimental recordings and/or manipulations to be targeted to specific cell types. This optimises experimental economy, meaning that the largest amount of information can be obtained from the fewest possible number of animals.

- Genetic studies of dementia patients have identified gene variants that cause dementia or increase dementia risk. Several lines of GA mice have subsequently been developed that express versions of these genes to drive expression of dementia-linked pathologies. These mice are therefore refined dementia models as they enable inferences about the effects of disease-relevant pathologies on mammalian neural systems. We will use dementia models that are well characterised in the literature, including by our own studies, and that are therefore known to recapitulate aspects of dementia that are consistent with the project aims.

Methods

Intracranial infusions

We will infuse three classes of substance into the brain:

- Reporters of cellular anatomy or activity

Infusion of these tools into the brain enables specific cell types to be visualised using microscopy. These markers are not toxic and enable targeted recordings to be performed from cell populations of interest, which increases the economy of the experiments. This means that the maximum amount of experimentally relevant information can be obtained from the fewest animals possible.

- Actuators of cellular activity

Infusion of these tools into the brain enables the activity specific cell types to be adjusted using either light stimulation or a non-endogenous drug (i.e. a substance that does not affect other cells in the body). By adjusting the activity of specific cells in a controlled manner, it is possible to test causal relationships between cellular function and memory ability. This ability to directly test causality optimises the construct validity of the experiment (i.e. how effectively an experiment tests the phenomenon being studied), meaning that fewer experiments (and therefore animals) are needed to draw reliable conclusions from the data.

- Proteins or viral-genetic tools that seed dementia pathology

Most GA models of dementia express protein-based pathologies throughout the brain. By infusing dementia-linked proteins or genetic tools to express these proteins in the brain, we can target the expression of a dementia-causing pathology to a specific brain area. This allows us to effectively model the earliest preclinical stages of dementia to understand

how local brain circuits are affected by pathology and study how the pathology spreads through the brain, like in human disease.
In vivo neurophysiological recordings

Recording from brain cells whilst animals are engaged in behaviours is necessary to understand neural mechanisms underpinning memory and thus routes to enhancing memory function in pathological states. This project will use two approaches for recording cellular activity in behaving animals: electrophysiology and optical imaging. Each of these methods generates different but complementary types of information about brain function. By tailoring our methodology to the experimental need (i.e. the specific hypothesis being tested) we can optimise the construct validity of our experiments (i.e. use the most accurate measure of the underlying concept we aim to measure).
Neurons signal to one another on very fast timescales (around 1/500th of a second) and coordinated neuronal signalling is a fundamental correlate of memory processing. This coordinated activity generates rhythmic patterns of brain activity (called neuronal oscillations) that occur up to hundreds of times a second. To record this fast activity, we will use implanted electrodes that are less than the width of a human hair; this small size minimises damage to the brain.

Changes in neural circuits that enable memories to be stored occur across extended timescales of hours to weeks. Similarly, memory loss caused by disease-linked pathologies develops over extended timescales. These longer-term changes can be accurately measured using optical recordings, where a glass window is implanted over the skull, or a small lens is implanted into the brain. An important advantage of this approach is that individual cells can be tracked repeatedly across extended periods of time (days-to-weeks). This facilitates a longitudinal study design, which reduces the impact of inter-animal variability and increases statistical power, meaning that fewer experimental subjects are needed.

Why can’t you use animals that are less sentient?

Some experiments in this project will be performed using ex vivo tissues (approximately 30%) or in terminally anaesthetised animals (approximately 10%). However, to achieve the aims of this project it is necessary to link brain dynamics to experience of the world and behavioural actions. There is currently no non-sentient system that can model this cognitive process and the behaviours that manifest it in a way that can be meaningfully compared to humans. This, however, is essential if we are to understand how episodic memory malfunctions in neurological diseases and discover treatments that work in people.

We collaborate with researchers using non-regulated invertebrate species amenable to genetic manipulation, like Drosophila, to screen cellular changes caused by dementia risk genes. However, non-regulated invertebrates are not suitable models for this project because their nervous systems do not contain circuits that are comparable in their organisation or complexity to those found in the human brain. Thus, circuits that generate cognitive abilities in people cannot be mapped to equivalent brain regions in non-regulated invertebrates. Additionally, the neurons that comprise these circuits often do not contain the same molecular machinery that mediates their function. For example, neurons in C. elegans do not contain voltage-gated sodium channels which generate the firing of action potentials by neurons in the mammalian brain. Due to these differences in circuit organisation and composition, it is also difficult to accurately model diseases of human cognition in these species.

Immature animals are an unsuitable model because they do not possess the behavioural sophistication required to measure cognitive abilities, like episodic memory. Additionally, because memory loss and dementia is typically manifest in adulthood, mature tissues are a more refined model of this condition than those derived from immature animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Surgical technique

- We will preferentially use volatile anaesthetics to facilitate swift recovery from anaesthesia.
- Body temperature and fluid balance will be regulated throughout anaesthesia to minimise physical discomfort upon recovery.

- Peri-operative analgesia will be provided using non-steroidal anti-inflammatory and/or opioid drugs. Analgesia will also be provided as needed in the days following surgery.

- After surgery, animals will be monitored daily until they have fully recovered. Signs of post- operative pain will be assessed using the grimace scale (Langford et al. (2010) Nature Methods. 7(6):447-9).

Intracerebral micro-infusions

- Any substance infused into the brain will be obtained from a reputable commercial supplier or collaborators that have established protocols for its use within non-toxic parameters. Based on experience, the genetic/molecular tools that we will use take 2-4 weeks to reach useful expression

levels in the brain. Longer-term expression does not cause adverse effects, meaning that animals do not suffer additional harm from longer incubation times.

- To minimise the potential harm of intracerebral infusions, we will only infuse small volumes of material (up to 5 µL), and this will be done slowly (<0.01 µL/sec), which the literature and our experience suggests does not cause damage by increasing intracerebral pressure.

Behavioural techniques

- In addition to reducing animal suffering for ethical reasons, our procedures are designed to minimise impact on animal wellbeing so as not to confound assessment of memory-related behaviours. The tasks that we use are sensitive assays of animal welfare, since even mild discomfort precludes engagement. Additionally, these tasks support animal health by providing environmental enrichment and exercise.

- Animals will be habituated to handling by the experimenter and any recording apparatus prior to behavioural testing. Additionally, to minimise stress from transportation from confounding behavioural measures, animals moved between facilities will have a minimum acclimation period of 7 days prior to use in behavioural or recovery surgery procedures.

• The tasks that we will use can be divided into two broad types: those that measure naturally occurring behaviours and those that require learning of task-associated behaviours.

a) Tests of natural behaviour do not require learning of task-related demands and are minimally stressful for the animal. Consequently, animals may be tested in one or more such tasks and perform an additional learning task.

b) Tasks that involve learning require an initial training period for the animal to learn the task demands. Animals will only experience one learning task, as conditioning to one task paradigm could confound behavioural measures in another task.

Neurophysiological recordings in head-fixed mice

• To image the brain in vivo, it is typically necessary for the head of the animal to be still under a microscope, and therefore head-fixation is required. Animals will receive a cranial implant that contains a head-plate that can be connected to the imaging equipment.

• To facilitate imaging in awake mice, the body of the animal rests on an apparatus that enables the animal to move and behave. This is either a passive (i.e. non-automated) treadmill that allows the animal to walk and run, or a tube shelter comparable to that used in the animal's home-cage as enrichment. Each of these behavioural paradigms are validated in the literature and we have extensive experience using them.

• Rats are larger and heavier than mice and are thus less easily integrated with the head-fixed behavioural paradigms that we have experience using. Consequently, head-fixation will not be performed in rats.

• It is essential that stress to the animal is minimised during head-fixation to avoid confounding the assessment of cognitive behavioural performance. In our experience, mice habituate effectively to

head-fixation when a staged approach is used. First, mice are acclimatised to the behavioural apparatus by freely exploring it (i.e. while non-head-fixed). Second, mice are head-fixed on the apparatus for an increasing amount of time per day, initially for less than 5 min, building in 5-10 min increments up to 20 minutes (typically 3 days). During head-fixation, mice may be given positively reinforcing food rewards such as sweetened water. Any animal that persistently expresses stress indicators such as struggling, a hunched posture, or vocalisations is immediately removed from head- fixation, and animals that do not progressively habituate across successive days are removed from the study. Habituated animals may then progress to training in a head-fixed behavioural task. Most head- fixed sessions are completed within 45 minutes and will not last longer than 75 minutes. This provides enough time to collect high quality data but limits the potential for adverse effects from prolonged head- restraint. The total number of awake, head-fixed sessions will be limited to 30, including habituation and behavioural training.

Dietary restriction

• For some behavioural tasks, it will be necessary to motivate task performance by placing animals on a controlled dietary schedule with provision of positively reinforcing appetitive rewards. This is necessary to reproduce a consistent state of motivation across

successive testing sessions to stabilise behaviour, which is requisite for valid assessment of cognitive abilities.

- In addition to motivating performance, provision of palatable rewards can reduce stress associated with initial habituation and training, since animals are provided with a reinforcing behavioural cue.

- Either food restriction or water restriction (i.e. mutually exclusive procedures) will be used, dependent on which constitutes the most refined method to motivate stable behaviour in a specific task. For example, food restriction can be the most refined method for tasks that involve searching (e.g. maze exploration) because solid foods can be readily dispersed throughout an environment and foraging for food is a natural behaviour for rodents. Conversely, delivery of liquid (via a lick spout) is a more refined reinforcement method for head-fixed and some freely moving tasks because the timing of reward delivery can be more precisely coupled to the animal's behaviour (e.g. location in a real or virtual environment), thus providing animals with more accurate behavioural feedback.
- Animals will always receive daily food and water in addition to that available during behavioural tasks. Animals on water restriction will always receive a minimum of 4 hours ad libitum access to water in the home cage per day. Evidence suggests that water deprivation for 24 hours does not induce physiological or behavioural signs of distress in mice and rats (Rowland. 2007 Comparative Medicine. 57(2):149-60; Bekkevold et al. 2013. JAALAS. 52(3):233-9) and that water restriction up to 22 hours per day across consecutive weeks does not cause adverse effects (Tucci et al. 2006. Behav Brain Res. 173:22-9).

- The body weights of animals on food or water restriction will be monitored daily to ensure that it does not drop below 85% of the free-feeding weight. If the body weight decreases below this threshold, food or water provision will be increased immediately. Published evidence indicates that rodents that lose up to 15% of their free feeding body weight through dietary restriction perform behavioural tasks stably and do not exhibit adverse physical or psychological signs (e.g. increased levels of stress hormones; lethargy) (Rowland. 2007. Comparative Medicine. 57(2):149-60; Perea et al. 2021. Front Vet Sci. DOI: 10.3389/fvets.2021.639187). Additionally, evidence suggests that calorie restriction can

improve long-term measures of health in rodents (Holehan and Merry. 1986. Biological Review. 61: 329-368) and reduce the incidence of behaviours associated with stress (Levay et al. 2007. Physiology and Behaviour. 92:889-96). Furthermore, stress impairs the memory ability of rodents (Moreira et al.
2016. PLoS One. 11(9):e0163245) suggesting that animals performing at criterion levels in behavioural tasks are unlikely to be stressed by dietary restriction.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the PREPARE guidelines to refine our experimental design (Smith et al. 2018. Lab Anim. 52(2): 135-141), the ARRIVE guidelines to refine reporting of our research (Percie du Sert. 2020.
PLOS Biology. 18(7): e3000410) and keep up to date with best practice information from the Laboratory Animal Science Association (LASA;
https://www.lasa.co.uk/current_publications/).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To identify possible refinements, I regularly review the research literature and resources from organisations that promote the importance of the 3Rs, like the NC3Rs (www.nc3rs.org.uk) and Norecopa (https://norecopa.no/about-norecopa). I also maintain a dialogue with the NC3Rs regional manager and academics using similar experimental models and techniques through collaborations and attendance at conferences. Additionally, I teach classes to undergraduates and PhD students that focuses on the design, reproducibility, and validity of animal studies, which requires that I regularly update my knowledge of best research practice. Where 3Rs advances are identified, we will attempt to integrate them into our practice in close consultation with our NVSs and NACWOs.

187. Impacts of Deer and Small Mammals on Ticks and Tick-Borne Pathogens

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Ecology, Animal Movement, Tick vectors, Infectious diseases, Environmental change

Animal types	Life stages
Roe deer	adult, pregnant, juvenile
Mice	juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand how landscape structure impacts space use by roe and fallow deer and small mammals, and how this affects risks to humans from ticks and tick-borne pathogens. This will inform predictive risk maps of tick-borne pathogens and environmental interventions to reduce disease risk.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Risks from tick-borne zoonotic diseases such as Lyme disease and Tick-borne Encephalitis virus are increasing rapidly in temperate regions, including the UK, and could be exacerbated by climate change and large-scale policy-driven changes in woodland connectivity. Risk mitigation requires that we better understand the ecological conditions allowing tick-borne pathogens to establish and spread and how these are modified by land-use and climate change. Our inter-disciplinary NERC funded project (TickSolve) focuses on key current and emerging pathogens threatening the UK, and will advance the evidence base for environmental mitigation of Tick-borne Diseases.

An important knowledge gap is understanding how landscape structure affects the movement of deer and small mammals and how this impacts zoonotic disease risks from ticks. Deer feed and move adult ticks between woodlands and other habitats and are critical in maintaining tick populations but do not transmit the pathogen causing Lyme disease (Borrelia burgdorferi) or Tick-borne encephalitis virus.
Small mammals can transmit both pathogens and are important hosts for immature life-stages of ticks.

In this project we will attach GPS collars to deer in study landscapes in England and Scotland which have been chosen to cover a gradient of woodlands of different sizes and separation from other woodlands. Within these study landscapes we are also collecting extensive environmental data and field data on tick density and wildlife host communities from camera traps. The data from GPS collars on deer will provide i) fine scale data on deer space use and behaviour in relation to tick distribution and ii) quantify landscape connectivity to deer movement across a range of woodland sizes and separation from other woodlands. We will also trap small mammals at these sites to measure their abundance, tick infestation and infection rates. The data from small mammal trapping will be analysed to i) see how rodent abundance relates to landscape metrics and ii) deer abundance and movement. To understand how woodland age impacts host communities, we will also trap small mammals at sites which include long term monitoring sites with extensive habitat, climate and host community data for bird and deer spanning a range of woodland ages.

We will assess how landscape connectivity to deer movement impacts tick density and infection in mathematical models which combine all of the wildlife host and tick data we have collected. This project will facilitate the design of more effective, better contextualised disease prevention strategies including environmental barriers to interrupt transmission.

What outputs do you think you will see at the end of this project?

At the end of the project we will have:

1. Quantified and published our research findings in peer reviewed journals on the impacts of landscape structure on roe and fallow deer distribution, habitat selection and behaviour using GPS collar data, and consequent impacts on ticks, Lyme Disease pathogens and disease hazard.

2. Developed, and published in peer reviewed journals, models which integrate landscape connectivity to deer with wildlife community (including small mammal trapping data), tick density and infection and human habitat use across the landscape, to (a) identify high risk areas for human exposure and (b) evaluate whether environmental barriers to deer and interventions may reduce human exposure.

3. Quantified and published our research findings in peer reviewed journals, on the role of landscape connectivity to deer and small mammals together with climate and habitat in restricting UK foci of tick- borne encephalitis virus at a landscape scale through tick sampling, deer GPS movement data and pathogen genomics.
4. Quantified and published our research findings in peer reviewed journals, on how woodland age impacts deer and small mammal communities to understand how woodland regeneration policies will impact tick-borne disease risk over time at the landscape scale.

5. Developed, and published in peer reviewed journals, models of the impacts of current and future changes in landscape connectivity and climate on arrival and emergence of TBEV and Hyalomma spp. ticks (which can transmit Crimean Congo Haemorrhagic fever) across the UK to better target surveillance.

6. Co-developed environmental intervention strategies, risk maps and communication materials to mitigate tick-borne disease risks with cross-sectoral stakeholders which consider barriers to deer movement.

7. With international experts, we will have scoped out the benefits of inter-disciplinary systems approaches for understanding environmental change impacts and mitigation options for tick-borne disease systems worldwide.

Who or what will benefit from these outputs, and how?

The outputs from this project will provide benefits to academics, stakeholders and policymakers in land management and public health and communities affected by ticks and diseases. We have surprisingly limited knowledge about how wild animals move within different landscapes – for instance to our knowledge there have been no past tracking studies of two of the most important hosts of ticks (roe and fallow deer) in the UK – and this limits our understanding of how pathogens and parasites spread in the wild. The project will generate significant academic benefits and development of scientific understanding and knowledge sharing. These benefits will be delivered both in the short-term during the project, and following completion of the project. We will have monthly team meetings among the interdisciplinary project team, annual international meetings with tickborne disease researchers and will regularly present our research at international conferences and publish the research methods and outcomes.

During the project, and following completion, researchers in movement ecology and landscape epidemiology will benefit from new understanding of links between landscape connectivity, habitat use and movements of diverse UK deer species (and data will be made publicly available via MoveBank). By linking this with pathogen genomics, phylogeography and models, we will advance theory linking parasites and pathogens to host dynamics at the landscape scale. Insights we gain will be applicable to a wide range of multi-host pathogens in which wildlife are key reservoirs. For deer, these include midge-borne bluetongue and Schmallenberg viruses, Chronic wasting disease and bovine tuberculosis.

During the project, and following completion, modellers, epidemiologists, ecologists and public health researchers will benefit from our innovation of incorporating landscape metrics, host and human habitat use into agent based, spatial, epidemiological models. This will underpin development of such models for other infectious disease systems to inform optimal mitigation strategies in complex changing landscapes e.g. risk-based Public Health surveillance.

The projects approach of co-developing research, models and risk communication materials with UK stakeholders, accounting for diverse land management priorities, will enable formulation of future- proofed woodland and greening policies in the longer term that minimise risks of these diverse tick- borne diseases.

How will you look to maximise the outputs of this work?

The project is an interdisciplinary collaboration between four institutions. There will be significant academic and science-policy outputs from this work.

Academic Outputs

The dissemination of new scientific knowledge will be maximised through publications and presentations at conferences. The projects approach of co-developing research, models and risk communication materials with stakeholders, who have different land management priorities (spanning conservation and biodiversity, recreation, forestry and deer stalking and climate mitigation), will enable formulation of future-proofed woodland and greening policies in the longer term that minimise risks of these diverse tick-borne diseases. Furthermore, engagement with key global partners and networks through webinars and meetings will facilitate transfer of the projects inter-disciplinary approaches from year one of the project to other rapidly changing tick-borne disease systems worldwide.

Science-Policy Outputs

The project has been designed with a One Health approach and will develop a science-policy interface for zoonotic disease mitigation with stakeholders. Using a participatory co-production process we will

integrate knowledge and priorities of key stakeholders at the landscape level, through workshops, focus groups and interviews throughout the project. We will co-develop risk outputs and policy briefs that combine their knowledge with the projects empirical and model outputs to maximise the impact from this work.

Species and numbers of animals expected to be used

- : 30
- : 20
- : 375
- : 375

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Wild roe and fallow deer have been selected as these species are among the most important hosts of adult tick life-stages in woodlands in the UK and understanding their movements and habitat preferences is necessary to understand how this affects risks from

ticks and tick-borne diseases. Adult deer will be selected to reduce any issues with the neck size of the animal growing during the study.
The movements of adult deer are most relevant to our research question, as adult deer movements are expected to have a greater impact on tick distribution compared to juveniles.

Adult and juvenile wild mice and voles of both sexes will be trapped as the most important hosts of immature ticks in woodland habitats. Adults and juveniles are important hosts for immature ticks and tick-borne pathogens.

Typically, what will be done to an animal used in your project?

Wild roe and fallow deer will be captured, a sedative or anaesthetic administered by intramuscular injection, a GPS collar attached to the neck, and an ear tag placed and ear notch made for identification. If anaesthetic is used, this will be reversed and the animal released and monitored for normal activity and behaviour visually and using the signal from the GPS collar. The GPS collar will be pre-programmed to detach after 18 months.

Wild mice and voles will be captured using traps which will be checked twice a day. Mice and voles will be restrained for a short period of time to weigh them, determine the sex of individuals, estimate the age, count and remove ticks on the head and ear, take a 2mm ear biopsy, blood sample and release. An area of fur will be clipped to prevent re-use of the same individual. Non-target species (eg shrews) and small mammals previously trapped will be immediately released.

What are the expected impacts and/or adverse effects for the animals during your project?

Deer

Traumatic injury during capture, skin laceration, antler damage (3-5 days for minor skin injuries to heal) Pain from intramuscular injection of sedative or anaesthetic (short duration, less than 10 minutes) Short-term low-level reduction in activity following capture and anaesthetic (up to one day).

Small mammals

Mild discomfort from ear biopsy and blood sampling.

Lethargy from trapping and restraint procedure in a small proportion of individuals (approximately 5%).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Roe deer

- Intramuscular anaesthetic injection, (100%), mild severity

- Traumatic skin or muscle injury, (5%) (estimated), mild severity

- Short term low level reduction in activity following release after capture and anaesthetic (100%), mild severity

Fallow deer

- Intramuscular anaesthetic injection, (100%), mild severity

- Traumatic skin or muscle injury, (10%) (estimated), mild severity

- Short term low level reduction in activity following release after capture and anaesthetic (100%), mild severity

Mice and voles

- Ear biopsy and blood sampling (100%) mild severity

-Lethargy and reduced activity (5%) mild severity

What will happen to animals at the end of this project?

- Kept alive
- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This study aims to understand the effects of environmental change on tick-borne disease risk. Government policy to expand woodland area and connectivity and climate change are highly likely to increase the risk to humans and animals from tick-borne diseases. Deer and small mammals are key hosts for ticks. Studies of movement of deer in relation to landscape structure (across a gradient of woodland fragmentation) and with respect other environmental barriers, eg unsuitable habitat, roads and rivers will help us understand how risk from ticks and tick-borne diseases may change in the future. The deer movement ecology studies and small mammal trapping will be carried out in study landscapes where we will simultaneously collect data on tick density and infection rates in the environment and work with stakeholders to develop locally appropriate environmental management solutions.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives. There is no existing data which could be used in mathematical models. The only existing datasets of GPS collar data from roe and fallow deer are from mainland Europe where habitats differ.

Why were they not suitable?

There is no existing roe or fallow deer movement data from the UK which span a range of landscape structures. There isn't a suitable alternative to small mammal trapping because it isn't possible to make predictions of how small mammal abundance, tick infestations and infection rates will vary across a range of landscape structures from existing data.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Deer

We will capture deer across two study landscapes. In each landscape we aim to capture at least 15 deer across areas categorised as having different landscape structures which will contain different size woodland patches and different amounts of open agricultural land. This is comparable to previous landscape movement ecology studies of roe deer (e.g. Coulon et al 2009 DOI:10.1007/s10980-008- 9220-0).

Small mammals

We will use a trapping grid with 100 traps at up to 30 sites for up to 5 trap nights, with an estimated 1- 10% trap success which will give an estimated 5-50 individuals trapped at a site. We estimate that these will be approximately 50% mice and 50% voles. This will provde robust data to parameterise mathematical models of tick-borne disease risk.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To reduce the number of deer needing to be captured in this project, we are collaborating with researchers in Europe who have collected GPS collar data from hundreds of roe deer through the EURODEER collaborative network (https://eurodeer.org/). These data can be used to inform predictions of habitat selection of deer in the UK which we then can test with the GPS collar data collected in this project.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As part of this project, we will analyse EURODEER network roe deer movement data and make predictions for distances of roe deer movement between habitat types within our study landscapes. The data from the deer we will capture and collar will be used to validate these predictions and to assess whether European roe deer movement datasets including GPS collar data from many hundreds of roe deer maybe applicable in areas of the UK with contrasting ecology. No UK deer movement data is held by the EURODEER network currently.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Roe and fallow deer are common wild deer species across the UK and Europe which often live near to areas of high human usage such as parks and gardens. These deer species are important hosts for adult lifestages of the tick which transmits pathogens causing Lyme disease and Tick-borne

Encephalitis so we need to study their movement to understand how this influences tick distribution and risk to humans from tick-borne diseases.

The capture methods will minimise harm to the animals by choosing the most suitable method for the environmental context and all people involved in the capture being trained, experienced and competent. The handling time for each animal will be minimised to the shortest possible time and animals will be monitored for full recovery from sedation or anaesthesia. Any animals which appear to be diseased or injured following capture will not be used in the study.

Trapping methods for mice and voles are well established and widely used, providing bedding and food and checking traps every twice a day (maximum trapping interval 16 hours) will minimise any discomfort experienced by the animals.

Why can’t you use animals that are less sentient?

We need to use wild deer and small mammals in our project as these species are the most important hosts for feeding life stages of the tick vector which transmits several important tickborne diseases in the UK including Lyme disease and Tick-borne Encephalitis virus. We need to collect deer GPS movement data to understand how deer habitat usage and movement influences tick populations and tick-borne disease risk in the UK. Rodent trapping data, tick infestation rates, blood and skin biopsies will give us important information on how these animals contribute as hosts for ticks and pathogen transmission.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will refine the procedures by increasing post-release monitoring of animals which show any signs of changed behaviour or in the unlikely event there are any injuries to animals captured.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will use published best practice guidance available on the National Centre for the Reduction, Refinement and Replacement Centre website https://nc3rs.org.uk/who-we-are/3rs
How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will keep informed of advances in the 3Rs through regularly consulting the National Centre for the Reduction, Refinement and Replacement Centre website https://nc3rs.org.uk/who-we-are/3rs

During the project we will take opportunities to refine our procedures to minimise welfare costs and will maximise the outputs from the data analysis to reduce the need for future capture and collaring of deer and small mammal captures.

188. Application of Ruminant Immunology for the Development of Novel Diagnostic Strategies and Vaccines Against Bovine Tuberculosis.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words
Bovine tuberculosis, Cattle, Diagnostic tests, Vaccination

Animal types	Life stages
Cattle	juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The main aim of this project is to generate the scientific evidence to underpin the UK government's control policies for bovine tuberculosis. These studies will focus on the development of diagnostic tests and vaccine strategies, and on the evaluation of potential shedding routes from infected animals.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The work contained in this project underpins the UK government's strategy for control of bovine TB. Work focused on BCG vaccination and the associated DIVA test (Detection of Infected amongst Vaccinated Animals) will allow the organisation and stakeholders such as devolved authorities (e.g. Welsh Government) the policy option of using a cattle vaccine as part of a strategy to control bovine TB in the National herd. Furthermore, performance data for the DIVA test will provide assurance to EU partners that the test is fit for purpose with regards to trade/export. In addition to supporting the government's vaccination strategy, we also aim to develop a defined antigen reagent as a replacement for tuberculin, with the objective of overcoming the shortfalls of using tuberculin reagents. Data generated from this project will provide additional confidence for the use of current diagnostic tests, while work identifying novel host biomarkers associated with bovine TB will address a requirement for improved diagnostic tools for bovine TB. Furthermore, understanding the shedding routes of M. bovis from infected cattle may inform potential control measures to limit animal to animal transmission.
Lastly, we will endeavour to define immunological correlates of vaccine induced protection, as such read-out systems could speedup development of other vaccine strategies if required.

What outputs do you think you will see at the end of this project?

Generation of scientific knowledge and dissemination through scientific publications and presentations at national/international meetings; development of improved diagnostic tests for bovine TB; data to support licencing and validation of diagnostic tests for bovine TB; data to support licencing of a bovine TB vaccine; increased understanding potential shedding routes of M. bovis from infected cattle.

Who or what will benefit from these outputs, and how?

GB government and industry stakeholders will benefit from improved diagnostic tests and a licenced bovine TB vaccine, as by reducing the prevalence, incidence and spread of TB in the cattle population, the financial burden posed by this disease on government and farmers will be reduced. A licenced bovine TB vaccine will also be of benefit to governments and stakeholders outside of GB where test and slaughter based control policies are not affordable/deliverable, and where vaccination could have a major positive impact on the TB control policies for these countries. Dissemination of the scientific knowledge generated in this project will benefit other researchers interested in ruminant immunology, TB immunology and TB vaccinology, including those working in human TB vaccine and test development programmes.

How will you look to maximise the outputs of this work?

Dissemination of new knowledge through scientific publications and presentations at national and international meetings; maintain and initiate collaborations with other recognised research groups in the field of bovine and human TB; ensure biological material from this project is archived and made available for suitable collaborations with other scientific and commercial organisations interested in diagnostic test development.

Species and numbers of animals expected to be used

Cattle: Cattle: Total of 940 (protocol 1: 300, protocol 2: 240; protocol 3: 400).

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Cattle are the target species for the diagnostic tests being evaluated in this protocol, and so their use is essential for this work. Evaluation of diagnostic test performance requires non-infected animals to estimate test specificity and infected animals to estimate test sensitivity. As well as using experimentally infected cattle, it is important that the diagnostic tests are performed using naturally M. bovis infected animals to ensure the data generated are applicable to control policies of bovine tuberculosis in the natural setting. Also, use of these animals is essential for understanding potential M. bovis shedding routes as a result of natural infection in cattle. Due to the complex nature of the immune response, vaccination studies remain the most appropriate and informative way to evaluate parameters of the immune response generated in cattle (the target species) following vaccination. Due to animal husbandry reasons, juvenile cattle will be selected for the majority of the studies in this project.

Typically, what will be done to an animal used in your project?

All animals on this project licence will undergo blood sampling from a superficial vein. In addition, nasal mucus, breath and faeces samples may also be taken via minimal evasive techniques, e.g. swabbing

of nasal cavity, wearing a breath sampling device and induction of defecation. Animals will typically be skin tested with tuberculin and other reagents containing recombinant proteins/synthetic peptides following the standard procedure for tuberculin skin testing. Some animals may be encouraged to swallow a temperature monitoring probe prior to skin testing. These procedures may be used in control animals, vaccinated animals or M. bovis infected animals. Eye examinations in some infected animals may be carried out, which would involve topical application of mydriatic eye drops. Animals used in vaccine immunogenicity and/or efficacy studies will be vaccinated with live attenuated vaccines or subunit vaccines, typically via the subcutaneous or intramuscular route, and some vaccines may require boosting with additional doses. Some studies will require the use of experimentally infected animals, either as a source of infected animals for diagnostic test evaluation or for determining vaccine protection. These animals will be sedated and then infected by installation of mycobacteria (typically M. bovis) into the trachea by endoscopy (generally once, but not more than twice in any experiment).
Finally, naturally infected cattle that have tested positive to routine bovine TB surveillance tests (tuberculin skin testing or blood-based interferon-gamma testing) will be selected, transported from the farm and housed in appropriate animal accommodation for typically 3 to 6 months. During this time they will undergo the procedures listed above (i.e. blood sampling; nasal mucus, breath and faeces sampling and skin testing). At the end of the study, animals will be transferred to a different project licence wherever possible, otherwise they will be killed using a schedule one method appropriate for cattle.

What are the expected impacts and/or adverse effects for the animals during your project?

From previous experience, the procedures listed in this project will cause no more than transient discomfort and no lasting harm. With regards to the use of naturally and experimentally infected animals, bovine tuberculosis is a chronic disease that develops over a period of many months/years, and clinical signs of tuberculosis are not expected in animals during the limited period these animals will be held under this project licence. No clinical signs of bovine tuberculosis have been seen in previous project licences covering this type of work.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mild - 100%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

For the development of TB diagnostic reagents as well as the vaccination strategies, no non-animal alternatives are available due to the need for a complete immunological response only possible in the live animal. The use of the target species (i.e. cattle) is necessary due to the variation between species. This is reflected by the fact that regulators (e.g. Veterinary Medicines Directorate) and international bodies (e.g. World Organisation for Animal Health) require data generated in the target animal for licensing or validation purposes.

Which non-animal alternatives did you consider for use in this project?

In vitro and in silico approaches.

Why were they not suitable?

No in silico or in vitro alternatives are available to test vaccine efficacy, define diagnostic parameters of ante-mortem tests, or to develop relevant biomarkers of protection or disease. However, we have cryo- preserved peripheral blood mononuclear cells (PBMC) from cattle undergoing regulated procedures as part of vaccination and/or infection experiments. These PBMC samples will be used to support some of our studies, such as initial immunogenicity screening experiments for novel test reagents.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Protocol 1: Numbers based on one experiment with control animals and one experiment with vaccinated animals each year of the project licence.

Protocol 2: Numbers based on performing one experimental challenge experiment and one vaccine/challenge experiment each year of the project licence.

Protocol 3: Numbers of naturally infected animals are based on: (i) the estimated number needed to provide the required precision in the estimate of test sensitivity of the DST-F reagent; and (ii) maintaining a source of naturally infected animals during the life of this project licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Group sizes in experiments will be determined using appropriate tools (e.g. NC3R's Experimental Design Assistant, Emory University's OpenEpi tool). In addition, statistical advice from the in-house biostatistician will be sought when designing new experimental studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have cryo-preserved peripheral blood mononuclear cells (PBMC) from cattle undergoing regulated procedures as part of vaccination and/or infection experiments. These PBMC samples will be used to support some of our studies, such as initial immunogenicity screening experiments for novel test reagents. In vaccine experiments, we will use where possible in vitro systems to prioritize the use of adjuvant in adjuvant/protein subunit vaccines. Furthermore, we will endeavour to maximise the outputs of studies in all three objectives by ensuring that relevant biological material from uninfected or vaccinated animals (protocol 1), experimentally infected or vaccinated/infected animals (protocol 2) and naturally infected animals (protocol 3) are collected and archived. Through our bio-archive project, these valuable samples will be made available to commercial organisations to help support diagnostic test validation exercises.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Vaccination of cattle: GLP safety studies have demonstrated that BCG is a safe vaccine for cattle with no or only minor local or systemic adverse reactions to be expected. For all vaccine studies, we will ensure that vaccine dose volumes are in accordance with established guidelines (Refining procedures for the administration of substances. Laboratory Animals, 2001. 35: 1-41).

Experimental infection of cattle: The method of low dose M. bovis infection via the endobronchial route is the most accepted and internationally used challenge method. We use an endoscope with animals under sedation, which significantly reduces the time of restraint and avoids the use of general anaesthesia. Endoscopy also allows the precise placement of the inoculum which improves the reproducibility of the pathology induced. Another major refinement has been the development of a protocol for infection in which cattle infected with M. bovis do not develop clinical signs of TB and we are still able to detect, at post-mortem analysis, differences in pathology between naïve and vaccinated animals.

Naturally infected cattle: Bovine TB is a chronic disease that develops over a period of many months/years, and clinical signs of tuberculosis are not expected in animals during the limited period these animals will be held under this project licence. No clinical signs of bovine tuberculosis have been

seen in previous project licences covering this type of work. However, all animals will be regularly monitored, and any showing signs of clinical disease (e.g. decline in general demeanour and body condition, laboured breathing, enlarged lymph nodes, difficulty feeding and loss of appetite) will be referred to a NVS and their advice followed (e.g. treatment, more frequent monitoring or euthanasia by a schedule 1 method).

Evaluation of blood-based diagnostic test performance requires blood sampling from a superficial vein, whilst evaluation of skin test reagents requires intradermal injection with a small volume of test reagent. Collection of samples for evaluation of shedding routes involve procedures with minimal invasive methods. From previous experience, we expect these procedures to cause no more than transient discomfort and no lasting harm. Where possible, the frequency of these procedures will be kept to the minimum required to achieve the objectives of the experiment.

Why can’t you use animals that are less sentient?

Cattle are the target species for the diagnostic tests and vaccine strategies being investigated in this project licence, and thus are the appropriate species to be used to achieve our stated objectives.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Pre-study meetings involving the NVS, NACWO and animal services staff will be held to discuss any advances in animal care. A discussion on the possible refinement of procedures will be a standing agenda item at post experiment wash up meetings with relevant individuals, including PIL holders and named individuals (e.g. NVS and NACWO).

As castration of male calves on farm may no longer occur, we will source young (juvenile) animals for the experimental studies in protocols 1 and 2 to avoid any potential behavioural issues. In addition, sourcing juvenile animals has the added advantage that these animals will be weaned on farm before being sourced for experimental studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow best practice obtained from NC3Rs, PREPARE and ARRIVE guidance.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By regularly checking information on the NC3Rs website, and regular communication with the local AWERB and relevant named individuals (e.g. NACWO, NVS and NIO).

189. Mechanisms and Applications of Targeted Protein Degradation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Protein Degradation, Chemical Genetics, Genetic Tools

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop genetic and chemical tools that enable drug-inducible protein degradation in mouse tissues. These tools will facilitate more refined and insightful animal experiments on a range of topics, and improve our understanding of the mechanisms through which proteins are destroyed.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The function of proteins is normally studied by mutating DNA and observing the consequences. However, DNA mutations are slow to take effect, which makes it difficult to distinguish the immediate and direct consequences of protein removal from downstream effects. DNA mutations are also difficult to reverse, which would be desirable to understand how therapies might work. Targeted protein degradation solves this problem by allowing proteins to be directly targeted for destruction using small molecules. This works very quickly, and can be easily reversed once the small molecule is removed.

Drugs which work via a mechanism involving targeted protein degradation are being developed in large numbers by the pharmaceutical industry. This approach allows drugs to be developed against targets that were previously considered to be undruggable. In the longer term, our work will provide better systems to understand how these drugs work in tissues, which should help to make them more effective.

What outputs do you think you will see at the end of this project?

This work will lead to fundamental information on how protein degradation mechanisms vary between different types of cells within tissues. This information will help to improve the design of protein degrader drugs for use in human therapies, and to develop more effective methods to understand the function of proteins in mouse models of disease.

Who or what will benefit from these outputs, and how?

In the short-term, the outputs will benefit researchers using mouse models to understand normal ageing and disease by allowing them to obtain more useful information from a similar or smaller number of animals. In the longer term, they will benefit the pharmaceutical industry and patients who stand to benefit from protein degrader drugs.

Targeted protein degradation involves a suite of cutting edge technologies that are not currently well used by the mouse genetics community. The knowledge developed under this license will feed into a service to be provided, under a separate license, by the UK national hub for mouse genetics. This will allow the benefits of our work to be shared by the broader mouse genetics community.

How will you look to maximise the outputs of this work?

Through our involvement in the UKRI National Mouse Genetics Network we are already collaborating with leading laboratories in the UK that use mouse models to understand disease and develop better therapies. We will disseminate our findings through collaborations with these groups that showcase targeted protein degradation approaches, and by running a training course at the national hub for mouse genetics. We will also present our work at national and international meetings and publish datasets from our studies regardless of outcomes.

Species and numbers of animals expected to be used

- Mice: 4000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is the most commonly used mammalian model organism. We aim to develop broadly applicable tools that improve how mice are used to understand human development and disease. All life stages will be used, from early embryos to ageing adults.

Typically, what will be done to an animal used in your project?

Animals used in this project will undergo administration of substances via injection, feeding, or surgically implanted drug minipumps, and be imaged under anaesthesia. In a small proportion of cases, these substances will cause cancer (e.g. colorectal cancer) to develop. Experimental durations will range from minutes to several weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Potential adverse effects include possible death under anaesthesia in survival surgeries, discomfort or pain following injections and surgical procedures, and toxicity induced during experiments to identify safe doses of novel drugs. Animals that develop colorectal tumours may experience rectal bleeding or diarrhea (up to 24 hours), anaemia and weight loss.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severities are considered to be moderate (~10% of procedures) or mild (~90% of procedures). For survival surgical procedures in juvenile animals, death from anaesthesia in and following surgery may occur more frequently. Death rates occurring under anaesthesia in juvenile animals will be monitored and reported.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We aim to develop tools for use in mice, which need to be tested in the animal where they will ultimately be used. We also aim to understand mechanisms of protein degradation in different cell types, for which the mouse is well suited due to the similar cell type organisation of many tissues compared to human.

Which non-animal alternatives did you consider for use in this project?

Cell lines and ex vivo primary cell cultures.

Why were they not suitable?
The chemical substances used to trigger protein degradation can be rapidly broken down by the body, and often do not enter tissues such as the brain effectively. These processes are very challenging to model in vitro; the best way to understand how molecules behave in a living animal is to test them in that environment.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We estimate the required numbers of experimental animals based on our past experience, usage statistics of previous publications, as well as information provided from other license holders at our establishment. The numbers required for breeding, maintenance and production of experimental cohorts are derived from estimates provided by the animal facility. To stay updated on optimized experimental design, we will regularly seek guidance from biostatistical experts employed by our establishments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We are developing a single transgenic line that enables us to evaluate at least 5 different chemical genetic systems for targeted protein degradation (degron tags), rather than developing separate transgenic lines for each system. We have estimated the required number of breeding pairs required to produce experimental cohorts based on the University of Zurich online tool (https://www.ltk.uzh.ch/en/Breeding.html). Pilot studies will be performed to assist with planning for any experiments requiring large cohorts. We will refer to the PREPARE guidelines for study planning and organisation. All experiments will be reported according to the ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will implement efficient breeding procedures and will additionally perform well-designed pilot studies whenever possible to assess the potential for publishable results. We will additionally perform computational modelling and analysis of datasets to maximize the information extracted from our datasets. Multiple tissues will be collected from each animal such that independent cohorts are not required to assess protein degradation in each tissue.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice as an animal model and determine which of several available methods for targeted protein degradation is most effective for different downstream experimental goals. These methods have previously been tested in mammals either minimally or not at all. Determining which methods cause the least harm is a critically important part of the assessment process, and will be a major output from this project.

Why can’t you use animals that are less sentient?

Mice are the model organism of choice for studying a wide variety of normal and disease processes, and this project aims to develop better experimental approaches specifically for this species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Determining which methods for targeted protein degradation have the lowest welfare cost is a critically important part of this project. We will implement pilot studies with increased monitoring the first time any novel substance is administered. Any studies involving surgical or invasive intervention will adopt appropriate pain management and post-operative care.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will stay up to date on best practice guidelines set forth and regularly updated from the NC3Rs website (Guidance on the Operations of ASPA - https://www.nc3rs.org.uk/).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about the advances in the 3Rs by attending informational events provided locally and provided by the National Centre for the Replacement, Refinement and Reduction of Animals in Research. Our involvement in the UKRI National Mouse Genetics Network will also provide a route to discover new 3Rs approaches, and to disseminate information from our own advances in this area.

190. Cell Therapy for Muscular Dystrophy with Genetically Corrected Cells

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Muscular dystrophies, Stem cells, Ex vivo gene therapy, Muscle development and regeneration

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to develop a new therapy for muscular dystrophies, genetic diseases affecting muscles which lead patients to progressive paralysis and premature death.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Genetic muscle diseases that appear in children affect a significant fraction of the population and essentially are treated with palliative, symptomatic therapies. No experimental therapy has shown to be clinically effective.

What outputs do you think you will see at the end of this project?

I expect to see scientific publications, at least one patent and a novel protocol to be tested in the clinics.

In addition, I will keep constant contact with patients' communities to update them on the progress of the work.

Who or what will benefit from these outputs, and how?

Humans and animals affected by muscular dystrophy will eventually benefit from these new protocols based on the results of this work.

Academic colleagues and collaborators will also benefit from the output of this work

By participating in the work to be carried out under this licence, students may benefit of the acquired knowledge on the disease and on possible therapies.

How will you look to maximise the outputs of this work?

The applicant has been working for several decades in the field and has developed a vast network of world-wide collaborators, who will contribute and advise within their specific competence in various aspects of the work.

Communication to scientific meetings and importantly with patients' advocacy group meetings will help to disseminate the output of the work as soon as we obtain robust and reproducible results. Publication of negative results will be considered when it may help others to avoid repeating unsuccessful experiments.

Finally, we aim at publications on scientific journals and dissemination to a lay audience with media engagement when enough evidence has been accumulated.

Species and numbers of animals expected to be used

- Mice: 4,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use mice because these animals represent a simple model of muscular dystrophy, which is milder than in patients; therefore the animals experience no suffering and only limping in the hind limbs around one year of age. Mouse and human anatomy and physiology are similar.

Typically, what will be done to an animal used in your project?

We will administer cells to dystrophic mice by intra-muscular injection. We may also administer drugs (e.g. anti-inflammatory drugs) alone or in combination with cells. Some drug (e.g. Tamoxifen) may be administered with an injection in the peritoneum.
In some case cells may be injected subcutaneously to test whether they may form tumours, in which case the animal would be humanely culled as soon as any tumour formation is detected. We will perform this protocol on 3 animals per subset and 4-5 subsets per year.

In some experiments biomaterials may be combined with cells and transplanted under the skin of the animals to test the formation in vivo of a novel bio-engineered muscle.

What are the expected impacts and/or adverse effects for the animals during your project?

The procedures to be used have been carried out for many years and usually do not cause major adverse effects. For multiple injections or subcutaneous implantation animals are anesthetised and treated with analgesic drugs. Tumours are not expected, and we do not observe severe adverse events such as pronounced limping or infection of the wound.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All procedures described above are associated with moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To develop a new therapy, it is necessary to design an experimental strategy that must be tested on healthy and diseased cells in culture, having clear and reliable endpoints that may inform of the toxicity and efficacy of a given treatment.

As it is not possible to experiment directly on humans and cell culture of muscle cells can only provide preliminary information, it is necessary to use the whole animal.

Which non-animal alternatives did you consider for use in this project?

We considered cell cultures, used for decades as first step before moving to animal work . Many groups, including our own, have tried to develop organ cultures of skeletal muscles,

that could faithfully mimic a normal, functional muscle, but so far these provide inadequate information.

Why were they not suitable?

Unfortunately, these artificial muscles reproduce only some aspects of muscle anatomy and physiology and, most importantly, only last few days in culture, thus preventing the study of long term effects.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have two main projects: Firstly, developing and tests new, more effective, protocols of ex vivo gene therapy in different mouse models of muscle diseases. The animals are studied with appropriate controls and the numbers determined by statistical advice to obtain meaningful information

The second project focuses on the origin of the development of the muscle cell in these diseases and requires fewer animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Previous experiences indicate that 5 animals is the minimum required to produce robust and reproducible information.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Different animals will be used in pilot studies to determine the appropriate strain and genetic makeup, to give us the most meaningful results. We will consult with experienced statisticians.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the most appropriate animal models for this project.

Why can't you use animals that are less sentient?

Mice are the most commonly used laboratory animal for their similarity to human anatomy and, to a lesser extent, physiology and their small size. The zebra fish model of muscular dystrophy is only useful for drug and genetic screening but is inappropriate for these studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We routinely administer anaesthesia and post-operative analgesia after any surgery or any potentially distressing interventions (such as multiple intra-muscular injection). In the many years that the anaesthesia/analgesia protocols have been in use, we have made refinements to the post-operative monitoring plan, including use of warming mats, more frequent observation until recovery, improved awareness of welfare indicators and communication between the responsible researchers and facility personnel.

The procedures of cell implantation will only cause only a minor discomfort and may be alleviated by the use of analgesic drugs. When multiple muscles are injected simultaneously, the animal will be anesthetised. In some experiments, artificial muscles will be implanted subcutaneously, by creating a pocket in the skin, that will be sewed after insertion of the implant. In this case, general anaesthesia will be followed by analgesic drug administration

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidance from BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinements and LASA guiding principles.

As well as continually consulting with experienced colleagues, there are several constantly updated publications on the web that can be easily accessed in case of doubts and to remain updated of the best practice to follow. For example,:

Guidelines for planning and conducting high-quality research and testing on animals (https://labanimres.biomedcentral.com/articles/10.1186/s42826-020-00054-0)

The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research (https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.3000410).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The nature of the planned experiments does not pose major concerns on animal welfare. Nevertheless, we will consult regularly with the personnel with experience of the NC3Rs and in consultation with the scientific community as well as NVS and NIO. Any advances in the 3Rs procedures that can be utilised will be promptly implemented in our project.

We are also signed up to NC3R newsletters and updates, which we use to review our protocols and projects.

191. Investigating the Effects of Obesity on Cardiac Structure and Function

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

obesity, heart, therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project licence will characterise the pathways by which obesity leads to cardiac dysfunction and to identify novel therapeutic interventions to prevent or reverse these changes in the heart.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The total weight of humanity is rising, and not just because there are more of us. Worldwide obesity has nearly tripled since 1975. Currently, nearly one-fifth of all humans are currently obese; in UK around 30% of adults are obese and in the United States this proportion exceeds 40%. Obesity contributes directly to development of cardiovascular disease and cardiovascular disease mortality. This is independent of other cardiovascular

risk factors, such as kidney disease, high blood pressure etc.. The mechanisms driving this are unclear. There is even less known whether obesity changes the cardiac structure and function in a manner that is different to other co-morbodities. Equally, we know little about how the standard and novel cardiovascular drug therapies affect obese cardiac tissue.

What outputs do you think you will see at the end of this project?

We will publish the data in relevant academic journals and present at national and international conferences. The studies may demonstrate utility of the therapeutic interventions for treatment of obesity and resultant cardiac structural, mechanical and electrical dysfunction.

Who or what will benefit from these outputs, and how?

Short to medium term we expect that the knowledge gained from this project will be shared with the scientific and clinical community and published in peer reviewed journals (output expected during the project). Thus the cardiovascular research community will be the primary benefactor of this work. The long term benefit of the work will be a better understanding of the mechanisms by which obesity affects the heart and whether these pathogenic mechanisms can be prevented using the standard or novel therapies. This will open new avenues to reduce cardiovascular disease risk for this patient population for example reducing atrial fibrillation or ventricular arrhythmias (output expected some 1-5 years after the project), with the ultimate aim to prevent or ameliorate high cardiovascular morbidity and mortality.

How will you look to maximise the outputs of this work?

Publication in the relevant scientific literature and presentation of the data at international meetings is likely to be the primary output for this work. This includes negative data. Existing collaboration with our clinical colleagues will also be further strengthened. The intention is that this work generates valuable datasets that will inform clinical practice and may provide a novel therapeutic avenue for the treatment of cardiac dysfunction in patients suffering with obesity.

Species and numbers of animals expected to be used

- Mice: 2000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the lowest mammalian species with sufficiently similar cardiovascular physiology to that of humans, allowing transfer of findings to man. We are however also conducting experiments in parallel in human cellular models and using human patient data.

Adult mice of a specific age range (8-20 weeks of age) will be chosen to minimise variability.

Typically, what will be done to an animal used in your project?

Some of the animals (less than 30%) will be fed a high fat diet to induce obesity. Mice aged between 6- 12 weeks will be fed high-fat diet and may also receive fructose solution in drinking water for up to 20 weeks. Some animals (less than 5%) may undergo minor surgery to implant an osmotic mini pump or equivalent device under the skin to chronically deliver drugs or vehicle control for up to 6 weeks. These animals will then undergo non-invasive assessment of structural and functional changes to the heart.
This will include echocardiographic imaging and ECG monitoring under anaesthesia before recovery. Blood sampling may also be carried out to measure any circulating factors influenced by the obesity or drug administration. In the majority (if not all of the animals), the heart will be excised under terminal anaesthesia for further Langendorff perfusion or cell isolation for assessment of cardiac and cellular function to maximize the data obtained from each animal.

What are the expected impacts and/or adverse effects for the animals during your project?

We will use wild type mice for high fat diet experiments or a genetically modified mouse line pre- disposed to gaining weight as an alternative to feeding with high fat diet (e.g. ob/ob mice or others). Genetically modified mouse lines will only be used for the therapy experiments, where a drug is known to prevent weight gain, such as SGLT2 inhibitors. These mouse lines are usually not expected to experience any adverse effects. However, if any of the mice develop unexpected phenotypic changes, the animals will be humanely killed.

Based on published data, the doses of the proposed agents to be delivered under this PPL have not been shown to cause any major or severe side effects in the mice or patients. Pharmacological agents may be administered either by mini-pump/rod implantation or intraperitoneal/subcutaneous injection or in drinking water.

The agents administered will not exceed the recommended maximum volumes for dosing. The safety profiles when injected subcutaneously or intraperitonealy in animals is well documented. However, if animals display unexpected adverse effects, then they will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

A large number of animals (30-40%) will undergo a non-recovery procedure, as they will only undergo heart extraction under terminal anaesthesia. The rest of the animals will experience moderate severity, due to cumulative effects of high fat diet or genetic manipulation, substance administration (minipump, injection(s)), echocardiography, in vivo ECG, and heart extraction under terminal anaesthesia.
However, every effort will be made to minimise any adverse effects.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cardiac mechanical and electrical function is complex, involving the interaction of multiple factors, including many cell types and physical and neurohormonal local controls, that cannot currently be studied without animal models. Our understanding of the processes involved, and their relative importance, limits our ability to use computer modelling, though this is a goal we are working towards.

Continued review of the scientific literature will be undertaken on a regular basis in order to identify any newly emerging technologies and models that could potentially be adopted in order to replace in vivo animal use.

Which non-animal alternatives did you consider for use in this project?

We have explored the use of stem cell-derived cardiomyocytes and human patient data. Work on stem cell-derived cardiomyocytes continues in my lab and we are currently replacing animal work where possible. For example we have successfully differentiated human induced pluripotent stem cells into cardiac myocytes and are starting to use these as alternatives to mouse primary adult cardiac

fibroblasts. We are also starting to use human patient data acquired during electroanatomical mapping procedures for the purposes of this and other projects.

Why were they not suitable?

Stem cell-derived cardiomyocytes, either in 2D or 3D culture, do not fully recapitulate the complexity of the heart, including many cell types and physical and neurohormonal local controls.

Human patient data acquired during electroanatomical mapping procedures is ususally acquired from patients that already have cardiac arrhythmias so whilst these are very valuable tools for studies, they do not allow for investigation of earlier stages of disease development.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Expected effect size will be determined through consultation of the literature, previous experience of techniques proposed or through small pilot experiments when possible.

We have used statistical methods to calculate how many animals we need to get meaningful data. This was determined based on the pilot data we have generated using our high fat obesity model and a number of different therapeutics and dosage concentrations we plan to investigate in vitro, on differential effects on atrial and ventricular chamber of the hearts.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When designing the experiments, we performed statistical analysis to ensure we use the minimum number of mice per group that will be informative. Importantly, we also employ a variety of approaches to reduce animal usage, including:

1. Using littermate pairs where possible to reduce variability of genetic and behavioural background

2. Performing and analysing experiments in a blind fashion where possible to reduce investigator- induced bias

3. Using standardised experimental methods to reduce variability

4. Optimising tissue usage through the use of innovative technology, such as RNAseq, optical mapping, novel cell isolation technique, etc..

5. The NC3Rs EDA tool will be used to design experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To maximise the information gained from a single animal we aim to perform multiple in vivo and in vitro analyses. Where possible, cell line work and in vitro manipulations have been designed to yield the maximum possible information and reduce animal use.

Better reporting of research should result in better science and more effective use of animals in experiments. Therefore, our findings will be reported (using the ARRIVE guidelines) in the scientific literature and at conferences, thereby minimising risk for future unnecessary animal experiments conducted by others.

Furthermore, literature will be continually reviewed to ensure that we are not repeating published work and that our hypotheses are based on the most up to date knowledge.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Majority of experiments are performed under terminal (non-recovery) anaesthesia so animal will only be aware of the induction of anaesthetic, and thus will not feel subsequent thoracotomy and heart extraction. We will continue to make efforts to refine protocols and further reduce the welfare costs. Current best practices (e.g. needle sizes used will be kept to a minimum, use of minipumps to deliver therapeutics) will always be followed.

The use of osmotic mini pumps to deliver the drug of interest over a long period of time (up to 6 weeks) reduces the need for repeated handling and injections and therefore reduces the distress caused to the animal. Analgesics will also be given pre- and post-operatively to ensure the least pain is felt by the animal following surgery.

Echocardiography will be carried out under general anaesthesia (up to 30 minutes) to prevent distress from physical restraint and equally to produce better quality data.

The apparatus for in vivo ECG/Blood pressure measurements is already refined to ensure the least distress to the animal i.e. tunnel used to secure animal in natural position and kept in dark throughout procedure. This experiment is done without anaesthesia.

Blood sampling will follow published guidance on suitable volumes which can be taken while minimising harms to animals.

In addition, before conducting each experiment using a novel drug (not previously administered), it will be discussed with the NACWO and NVS to ensure animal welfare is maintained throughout the experiment and that minimum suffering is caused to acquire the scientific endpoints. We will also review each experiment on completion to see what lessons can be learned from the study in terms of endpoints (scientific and humane) and any animal welfare issues that may have arisen during the experiment that could then guide the next experiment.

Why can’t you use animals that are less sentient?

The mouse is the least sentient mammal that is most similar to humans in terms of the way their heart works. For example, zebrafish is starting to get used more often but it only has one ventricle instead of two like the human heart. This prevents experiments examining chamber specific differences in terms of obesity of indeed drug therapy effectiveness.

Majority of our work will use animals that are terminally anaesthetised. However, experiments studying the long terms effects on the heart cannot be done in terminally anaesthetised animals. For some experiments we will also use cellular models of disease however, these cannot completely replace the need for the use of mice as they cannot replicate the complexity of the whole animal physiology.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Majority of animals will be used for non-recovery work so will only ever experience minimal suffering. This is possible due to our state-of-the-art optical mapping technologies, allowing for monitoring of heart electrical activity ex vivo.

To minimise the welfare cost to the animals, we will use good animal husbandry, and will provide enriched environment in the cage to keep the animals happier. We will also use

refined handling techniques to reduce the distress that animals experience during handling.
For all procedures, the mice will be carefully monitored and if adverse events are observed, monitoring will increase in frequency and steps, steps will be taken to alleviate them or the affected mice will be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the PREPARE guidelines to ensure our experiments are planned and conducted in the most refined way. LASA guidelines will be consulted to ensure correct dose volumes are selected for the specific route of injection/blood sampling. We will also stay up to date with the NC3R website resources and guidance on the best and most refined practices. All the data will be published according to the ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Regularly monitor the guidance given by the NC3R's website, making use of the online resources to ensure the project is carried out efficiently and using the best methods for animal welfare.

192. Parasite Epidemiology in Wild Bird Populations

Project duration

5 years 0 months

Project purpose

- Basic research
- Research aimed at preserving the species of animal subjected to regulated procedures as part of the programme of work

Key words

birds, wildlife, disease, malaria, trichomonas

Animal types	Life stages
Wild birds	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this work is to improve our understanding of how parasites interact with their wild bird hosts. We want to understand how variable the associations between parasites and hosts are (i.e. whether parasites can infect different hosts in different locations, and how this varies over time), how changes in the environment – such as changing food availability or climate – affect parasite infection, and how this can be managed for species conservation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The proposed work will provide novel insights into how parasite infections fluctuate within and between individuals and species in wildlife populations. The work will look at both vector-borne parasites such as avian malaria, and directly-transmitted parasites such as Trichomonas gallinae. The work will look at how parasite transmission is affected by the

distribution and availability of food resources, and will have practical implications for management of declining wildlife

What outputs do you think you will see at the end of this project?

This work will result in a greater understanding of the dynamics of parasite infections and health in wild birds, including implications for their conservation. Data and analyses will be published in peer- reviewed journal articles. The work will also result in advice for managing outbreaks of disease in wild bird populations, as well as a greater understanding of what drives these outbreaks.

Who or what will benefit from these outputs, and how?

Researchers in the fields of disease ecology will gain knowledge from the advances this work will make in the broad field. Students will benefit from the ability to conduct undergraduate and postgraduate projects using samples collected under this licence, as well as contributing (quality controlled) data to the research programme and thus being listed as co-authors on publications and outputs.

Conservationists will benefit from advice on managing disease in wild populations, likely to result from the hawfinch element examining how parasite strains vary over time and space.

The public will gain an increased knowledge and understanding of health and disease in wild birds from communications following outputs from the study of parasite epidemiology in a wild swan population, which has a citizen science and public engagement element as part of a wider project.

Full benefits are unlikely to be fully realised until the end of the project.

How will you look to maximise the outputs of this work?

All data from this work will be submitted for publication in peer-reviewed journals, including non- significant results and unsuccessful approaches where this is feasible. All molecular data from parasites will be submitted to open access repositories for genetic data, such as GenBank, where it will be accessible to other researchers.

Work with mute swans forms part of a wider project, examining swan health and welfare in relation to human-animal interactions, meaning that the data collected on health and parasite infection can also be used in association with other observational datasets collected from the same individuals.

Species and numbers of animals expected to be used

Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Birds are abundant, widespread, and diverse, and harbour a remarkably wide range of parasites, but we know surprisingly little about the spatiotemporal dynamics or epidemiology of infection.

Typically, what will be done to an animal used in your project?

Birds will be caught in the wild using standard techniques appropriate to the species, such as mist nets. They will be ringed using an individually numbered metal ring, have measurements taken, and be assessed for health status. If deemed healthy, they will have a small blood sample taken and they may also have an oral swab taken, to screen for parasites. They will then be assessed for health status again, before being released back into the wild.

What are the expected impacts and/or adverse effects for the animals during your project?

The protocols involved in screening birds for parasites are widely used and are known to cause minimal harm. All samples will be collected from each bird within a short space of time, at the capture site. Each bird will then be re-released into the wild without delay. No adverse effects are considered likely.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The maximum severity expected from the procedure is expected to be mild, with no negative effects beyond transient discomfort during the procedure.

What will happen to animals at the end of this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We wish to examine the dynamics of parasite infections in wild populations, so we cannot carry out this work without using wild animals in their natural environment.

Which non-animal alternatives did you consider for use in this project?

We considered non-animal systems, laboratory-based systems, and computer simulations.

Why were they not suitable?

The objectives of our work make it impossible to study either a non-animal system, a laboratory-based system, or use computer simulations. As the birds we study need to be

exposed to natural levels of disease vector exposure, and environmental stressors in order to achieve our objectives, a captive population cannot be used for this work.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In order to gain an accurate estimate of disease prevalence and how this fluctuates within and between individuals, we need to collect samples from a relatively large number of individuals across multiple species. This is for two reasons: first, because we need to have confidence in our prevalence estimates, because we know that parasite prevalence can vary markedly between different species, and we need to be confident that we have detected all parasite strains present within each species.
Second, because we need to control for environmental variables in statistical models to control for variation caused by these effects and to detect variation caused by the factors we are examining.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The nature of our work means we need to use relatively large numbers of animals. However, the protocol we are using only involves taking a small blood sample from a wing vein. Some birds will also have their throat swabbed. Both procedures are classified as mild and will cause only temporary discomfort to the birds during the procedure. All birds are expected to be released back into the wild at the capture site shortly after samples have been taken, once their health has been deemed satisfactory.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where possible, we will minimise sample sizes through the use of statistical models to control for background variation (in e.g. age, sex, time of year) during analysis.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are using species of wild bird within which previous work has identified that the relevant parasites are present in a large enough proportion of individuals within natural populations, that we can achieve our objectives.

The protocols followed in order to screen birds for parasites (a blood sample, and an oral swab) are well-established, and known to cause minimal harm.

Birds will be captured and handled by experienced individuals with the relevant licences, and will be released as soon as the required samples and measurements have been taken

Why can’t you use animals that are less sentient?

We wish to understand the dynamics of parasite infection in wild bird populations, so we need to study wild birds in their natural environment.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The protocols followed in order to screen birds for parasites are well-established, and known to cause minimal harm. If any adverse effects are seen, then protocols will be reviewed and revised accordingly. All PIL holders working independently (i.e. also holding bird ringing licences) will work alongside other bird ringers at least once per year, to ensure any refinements in bird capture and handling are incorporated into standard practice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will continue to adhere to guidance from professional bodies such as the British Trust for Ornithology to ensure birds are caught and processed in the most efficient way from a welfare perspective.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will engage with any relevant continuing professional development opportunities where possible.

193. Factors Regulating the Skin Immune System in Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Skin, Inflammation, Regulation, Tolerance, Immunology

Animal types	Life stages
Mice	embryo, neonate, adult, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify factors which regulate skin immune responses and to understand how these factors fulfil this function.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chronic inflammatory skin diseases such as psoriasis and atopic dermatitis/eczema are very common, can have a debilitating effect on people’s lives and they place large burden on healthcare systems.

Skin inflammation, exemplified by redness and rashes, is also a very common reaction to drugs and infections, which in some case can be serious, yet such reactions remain poorly understood.
The skin is a crucial barrier between the internal body and the environment, and as such it is home to a vast array of immune cells which are tasked with preventing infection by microbes. However, these same cells must remain unresponsive to the harmless microbes living on the skin and to harmless environmental substances which are constantly being encountered. Therefore, skin immune cells must be strictly regulated to prevent inflammation and immune responses occurring inappropriately.
Regulation is also key to ensure that inflammation is resolved appropriately after an infection has been cleared, as failure to do so will result in inflammatory skin disease.

Despite the prevalence and impact of inflammatory skin diseases, our fundamental understanding of the factors causing such disease are not well understood, and little is known about how inflammation is regulated and resolved. This lack of understanding impedes the development of new therapies, as appropriate mechanisms to target are not known.

In the past 10 years there have been huge improvements in treatments for moderate to severe psoriasis and atopic dermatitis however even these therapies do not cure disease and they also have significant side effects, they don’t work in all patients, and some patients develop reactions to the therapies themselves over time. Therefore, there remains an unmet clinical need for new therapies.

This project will identify factors regulating the initiation and resolution of skin inflammation and will examine mechanistically how these regulatory factors work. This will improve our fundamental understanding and will also identify novel factors which can be used in the future as targets against which new drugs can be developed for treating inflammatory skin diseases. The regulatory factors identified may also have roles at other sites in the body, so this work may be applicable to inflammatory diseases at other tissue sites, particularly those sites which contact the external environment such as the gut and lung where the regulation of inflammation is also critical.

What outputs do you think you will see at the end of this project?

This work will lead to publications detailing how skin inflammation is regulated in mice and how the resolution of inflammation is controlled. It will identify important factors in the regulatory process which may be future targets against which to develop new therapies to treat inflammatory skin diseases.
Importantly, it may also determine factors which are not useful targets for regulating skin inflammation.

Who or what will benefit from these outputs, and how?

In the short term this research will benefit immunology researchers by providing a better understanding of factors which regulate inflammation in the skin. These findings may also be directly relevant for other body sites, particularly the gut and lung where there is direct contact with the environment and microbes. Therefore, this work will impact researchers studying inflammation in the lung and gut.

This research will also benefit dermatologists as it will provide new understanding of the factors contributing to skin inflammation. In the long term we anticipate that this research

will lead to the development of new therapies which will allow dermatologists to have improved treatment options for their patients.

The project will benefit drug developers at pharmaceutical companies as it will deepen our understanding of factors that regulate skin inflammation and will identify new factors. This will identify new targets against which therapeutics can be developed for the treatment of inflammatory skin diseases. These targets may also be relevant for the treatment of inflammation at other body sites, so this research may have wide ranging implications for treatments for inflammatory disease across many body sites. Identifying novel therapeutic targets benefits the pharmaceutical industry as they can develop therapies which target these factors, leading to new therapies.

The research undertaken will determine how the skin immune system is regulated which will determine new therapeutic targets. We anticipate that this advance will lead to the development of new therapies for inflammatory skin diseases such as psoriasis and atopic dermatitis. Therefore, in the long term we anticipate that this research will lead to improved treatment options for patients with inflammatory skin diseases.

How will you look to maximise the outputs of this work?

We will disseminate the findings of this research through publications in open access journals, press releases and presentations at national and international conferences. We will ensure that the findings of this work reach the widest possible audience by presenting the research at conferences attended by both basic researchers and health care professionals.

We will collaborate with researchers in dermatology to begin to translate the findings of this research, and we will also collaborate with researchers examining inflammation at other body sites to determine if the factors that we find to be important for the regulation of the skin immune system are also involved in regulating the immune system in the lung or gut.

In addition to publishing findings identifying factors that are involved in regulating the skin immune system, we will also publish any findings which determine factors that do not regulate the skin immune system. This is critical to ensure that other researchers can gain insight from our data and will not repeat the same approaches that we have already used unnecessarily. It is also essential information for the development of therapeutics, as it may aid the identification of factors which do not regulate the skin immune system but, may regulate the immune system at other body sites, which may be an advantage for some therapies.

Species and numbers of animals expected to be used

- Mice: 7500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project requires the use of mice so that we can identify factors which cause (or prevent) disease and can distinguish these from factors which are merely changed in the diseased state, but do not directly affect the disease. The use of mouse models of disease also allows us to determine how these factors work, which is important as this can highlight other factors involved in disease. The use of mice allows the control of variables such as genetic differences and environmental exposures which cannot be easily controlled for in humans.

Working with human samples allows the identification of factors which are altered in disease relative to healthy individuals however, such approaches cannot distinguish between factors driving or preventing disease, and those which are an outcome of the disease, unless clinical trials are undertaken. To undertake clinical trials, prior knowledge and data are required suggesting that the anticipated outcome will be beneficial to patients. For example, we need to have evidence that a drug treatment targeting a particular factor will likely have a beneficial effect for patients, and it is not usually ethical to treat patients with a drug treatment that we suspect will induce or significantly worsen disease. Therefore, research is required in disease models before we can progress to translational research and clinical trials.

Whilst some research can be performed on human skin samples taken from volunteers and grown in the lab, these suffer from an inability to recapitulate human inflammatory skin disease (such as psoriasis or eczema) due to the inability to recruit immune cells from the blood stream into the skin tissue, which is an important stage in the initiation of inflammatory skin disease. Also, although some immune cells can survive for days in the lab, others are unable to survive and do not behave as they would in the body. The use of human skin samples also has drawbacks as there is high genetic variability between individuals and it is difficult to control for additional variables such as the level of exposure to sunlight, environmental chemicals and microbes, and variations in the disease severity

etc. Therefore, to robustly identify factors involved in driving or preventing inflammatory skin diseases, animal models are required where these variables can be controlled.

One of the most well used species for immunology research is the mouse, which has similar immune cells to humans and where well-characterised research tools are available to allow us to study the immune system. Mice are also a particularly good species to use as we can use genetically identical animals to accurately identify the effects of treatments on disease severity, and we can also use existing or newly generated genetically modified strains to allow the determination of the role of specific factors in driving or regulating disease.

In this research we will use adult mice where their immune system is fully developed. This will mirror psoriasis, as this disease largely affects adults. Although atopic dermatitis/eczema is more prevalent in the young, we will also use adult mice for atopic dermatitis models, as we can induce similar immune responses in adult mice to those seen in atopic dermatitis, so this is an appropriate approach which will allow us to interrogate regulators of the disease process whilst ensuring that the severity of our models is minimized.

Typically, what will be done to an animal used in your project?

In this research we will use adult mice to model the inflammatory skin diseases psoriasis and atopic dermatitis (eczema). These models involve daily treatments to the skin surface,

or injections to induce inflammation which resembles these diseases. This typically occurs by anaesthetising the mice, taking skin thickness measurements using callipers, then treating the skin surface with a liquid or cream, or with an injection into the skin with an agent which modulates a specific part of the immune system.
Treatments are typically performed daily for up to 10 days (but usually 7 days or less). In some cases, the resolution of inflammation will be examined by treating the mice to induce inflammation, then ceasing treatment and monitoring the clearance of inflammation.

Prior to, during, or after the induction of inflammation some mice will be treated with immune modulating agents or cells to allow us to determine the effects of each factors on inflammation. This may be via injections or the diet or via the use of genetically modified animals. We will also treat some mice with light radiation to label cells within the skin to allow us to track cell migration into and out of the skin tissue. This may be performed prior to, during or after the induction of inflammation.

Mice will be treated a maximum of once per day and all treatments and measurements will be done under a single period of anaesthesia, unless they can be humanely performed without anaesthesia. On occasion mice will be anaesthetised purely to measure the inflammation in the skin using callipers, as this is cannot be performed accurately without causing distress unless anaesthesia is used. This will be kept to a minimum and each mouse will be anaesthetised a maximum of 15 times in each experiment, although in most cases this will be 7 days of anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Psoriasis and atopic dermatitis (eczema) models.

The application of Aldara cream to the skin surface to model psoriasis causes weight loss, typically around 10% of the starting weight, but it does not usually cause dehydration or other overt signs that

the mice are unwell. The local skin becomes reddened, scaly and thickened which are hallmarks of psoriasis. Some inflammation in other parts of the body are observed in this model involving spleen enlargement and immune cell changes, but obvious signs of disease, such as a hunched position are not typically observed.

The administration of the vitamin D3 analogue, MC903 to the skin surface causes atopic dermatitis-like skin inflammation, involving local skin reddening, scaling and thickening and weight loss of typically around 10-15%. Similar to the psoriasis model, we do not expect to observe dehydration, or other overt signs that the mice are unwell in this model.

Skin inflammation may also be induced by treating the skin with immune modulators (cytokines or chemokines), which are small proteins that direct immune responses). We expect to observe reddening, scaling and thickening of the skin at the application site, but we do not expect to see dehydration, or other overt signs that the mice are unwell. Mild weight loss may be observed in this model, typically less than 10%. In some cases we will need to perform tape stripping, where the outer most layers of the skin are removed using adhesive tape. This itself causes mild damage to the outer layers of the skin, resulting in redness, but it is not expected to result in weight loss or any other adverse effects.

Typically ear skin will be treated, but if scientifically necessary then back skin will be treated.

For all of the skin inflammation models, if weight loss nearing 20% is observed, or the mice appear dehydrated, or show other overt signs that they are unwell, the mice will be humanely euthanised. Skin inflammation is expected from around 2 days after the initial treatment, for the duration of treatment and for up to 3 weeks after the cessation of treatment, whilst the inflammation is resolving. Typically, skin inflammation experiments will last for 7 days, so mice will undergo 5 days of inflammation. Where the resolution phase is being examined mice will experience skin inflammation for up to 3 weeks.

The radiation that we use to mark cells in the skin is at the boundary of UV and visible light and has the capacity to cause burns if used from a high intensity source or if the exposure occurs over a long period of time. However, we will use a low power ultraviolet source to minimize the risk of this occurring, and we typically irradiate for 5 minutes. Mice will be checked immediately following irradiation, and the following day for signs of skin tissue damage. In the unlikely event of this occurring, mice will be euthanised.

Treatments with diphtheria toxin can cause weight loss and toxicity if used in high doses or for a prolonged period of time. Pilot experiments will be used to optimise the dosing regimen for diphtheria toxin treatments to ensure that the most appropriate method is used with the least severe side effects. Other procedures such as treatments with cells or immune modulating agents are expected to cause no more than transient distress and no lasting harm.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

These models are to study skin inflammation, so will necessarily cause a number of days (typically 7 days, but up to 3 weeks) of inflammation which will be moderate in severity. Most animals treated will develop skin inflammation, but around 10% will not because they are either control mice, or the immune modulatory intervention given to them will be successful in reducing the inflammation.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Immune responses involve multiple cell types interacting in the context of a structured tissue architecture complete with a host microbiome. Therefore, although individual aspects of an immune response can be modelled in vitro, to identify novel factors regulating immune responses, models are required where the tissue structure and many

types of immune cells are present. Ex vivo human skin biopsies are one approach, but these lack the ability to recruit cells into the tissue, and many immune cells do not survive well in these models. Therefore, the most valid approach is the use of an animal model where all of the relevant immune cells are present and/or can be recruited and the cells interact in the context of the tissue microenvironment.

Which non-animal alternatives did you consider for use in this project?

To study human skin, 'skin equivalents' can be used where structural skin cells (fibroblasts and keratinocytes) are grown in the lab and become organised into a skin-like structure.

The other model system that we considered is using human skin biopsies which can be grown in the lab and treated to examine skin immune responses.

Human and mouse laboratory cultures of skin cells will be used where appropriate alongside animal models to allow us to test the effects of each factor on immune cell activity prior to its use in mouse models. This will allow us to replace skin inflammation experiments on live mice (protocol 2 or 3) with laboratory culture models where we will examine the effect of the factor under investigation, on the activity of a specific immune cell type.

Why were they not suitable?

Human skin equivalents are a promising approach however, they currently lack the ability to include most of the immune cells which are the drivers of inflammation and are the main focus of our research. Therefore, skin equivalents are currently not a useful approach to use for this research.

Similarly, the culture of human skin biopsies do not support the survival of many immune cell subsets and non-skin-resident cells cannot be recruited, again limiting the utility of this approach for skin immunology research.

Some cell culture approaches will be used which will replace some animal use, but it will be necessary to test some factors in animal models as well as in culture systems where there is the full range of cells present in the correct tissue architecture.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We typically use 6 mice in each experimental group to ensure that we can robustly answer our research questions whilst minimising the number of mice required. We estimate that we will use up to 200 mice a year for psoriasis model experiments for the 5-year duration of the project, which equates to up to 1000 mice. We also require similar numbers for the atopic dermatitis model.

Inducing skin inflammation by the use of immune modulators (cytokines and chemokines) will be used less frequently, therefore, we will use up to 500 mice for these approaches.

To label cells, delete certain cells, or examine how immune regulatory factors work, transgenic mice will be required. Wherever possible we will breed these to give both transgenic, and control mice within the same litter to reduce variables such as cage-to-cage variation. This increases the number of mice generated that are not useful (the incorrect genotype), so increases the number of transgenic mice generated, but it does reduce the variability and therefore reduces the number of mice undergoing procedures. Therefore, we will generate up to 5000 transgenic mice during this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We consult with statisticians to optimise our experimental design prior to starting experiments and through the project at appropriate intervals.

To reduce the number of mice required, we analyse multiple aspects of skin inflammation in the same group of mice. One example of this is scoring the skin inflammation daily, which allows us to track the severity of inflammation so that the most appropriate day to end the experiment and analyse the skin inflammation is evident.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For some mouse strains the most efficient breeding strategy where all mice are useable (have the required transgene or are required controls) is breeding the controls separately to the transgenic mice. However, this approach is not optimal as even maintaining strains using the best practice can result in mutations, and also the mice have different mothers and are separately housed, resulting in multiple unwanted variables. To remove these, wherever possible mice will be bred so there are transgenic and control mice in the same litters. However, this approach can also generate a significant number of unusable mice (e.g. heterogygotes). We will use littermate controls whenever possible therefore, our breeding will not be the most efficient approach, but it will lead to less variable data, reducing the number of mice which undergo procedures.

To ensure that our experiments have optimal sample sizes, power calculations have been performed. However, once we have a better idea of the variability of inflammation in each new strain of mice, we will adjust the power calculation to ensure that an appropriate number of animals is used and therefore mice will not undergo any procedures unnecessarily.

When using new treatments, pilot studies will be performed to optimise the dosing regime, thus ensuring that the optimal protocol is employed, reducing the use of mice.

To reduce the numbers of mice required we will take multiple tissues from each animal, ensuring that a panel of outcomes are measured, so that fewer additional experiments are required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use published skin inflammation models induced using Aldara cream, MC930/Calcipotriol and cytokines such as IL-23. These models work by activating the skin immune system and thus inducing inflammation, rather than causing damage to the skin. The previous best model for psoriasis used immunodeficient mice and involved grafting them with non-lesional human skin from psoriasis patients. The skin inflammation models used here will cause less pain, suffering and distress than this xenotransplantation model as surgery is not involved in the models we will use. The models we will use are also better models of the immunology of psoriasis, as they are driven by host inflammatory responses and are not directed against foreign material, as is seen in the xenotransplantation model.

Any skin inflammation model will need to induce some level of suffering as inflammation is required, but the models that we will use are induced by methods (topical or intradermal treatments) which themselves do not cause lasting pain or harm to the animals.

Why can’t you use animals that are less sentient?

This research is examining factors regulating inflammatory skin diseases. Therefore, models are needed which have a similar immune system and skin to humans to enable the research to be easily translated. Therefore, mammals are required, and mice are the most appropriate species due to their small size and the existing knowledge and tools to modulate genes, cells and immune responses which will allow us to determine mechanisms involved.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will work to speed up taking measurements and applying the inducers of inflammation, so that mice spend the minimum amount of time under anaesthesia. We already check the mice on recovery from anaesthesia, and around 10 minutes after recovery, but we will add in additional checks if required in future.

We will refine our protocols by reducing the number of days that the mice are treated for where our experimental readout is an immune cell type or activity which is important for the induction of inflammation. To determine the optimal day for analysis we will perform pilot experiments and will then choose the shortest treatment time which will enable us to answer the research question.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow best practice guidelines such as PREPARE (Smith AJ et. al.; PREPARE: guidelines for planning animal research and testing Lab Anim. 2017, 52(2):135-141), those published on the NC3Rs website and in peer reviewed publications such as Morton DB, 2001 (Morton DB et. al.; Joint Working Group on Refinement. Refining procedures for the administration of substances. Lab Anim. 2001, 35(1):1-41). We also receive alerts from Pubmed when new skin inflammation papers are published, and we discuss these in fortnightly journal club meetings. In particular, we discuss how the experiments were performed, and if this has any implications for how we perform our inflammation models, with a view to incorporating any refinements in our protocols.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We stay informed about 3Rs advances through posters and newsletters sent by the animal facility, and through the NC3Rs newsletters and attendance at NC3Rs symposia. We implement new advances by holding regular group lab meetings dedicated to animal work, where we discuss any issues with the colonies, discuss possible refinements to procedures, and plan how to implement any advances in the 3Rs.

194. Genetic and External Influences on Regulation of the Immune System

Project duration

5 years 0 months

Project purpose

(a) Basic research

Key words

immune system, infection, inflammation, radiotherapy

Animal types	Life stages
Mice	neonate, embryo, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to understand how the immune system functions to maintain health by protecting us from infection and damage, but how this sometimes goes wrong and causes disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

When our body becomes damaged, it is crucial that our immune system responds quickly and appropriately to keep us healthy. An example of such damage is when we are infected by a harmful pathogen like a bacterium or virus. Another example is when we receive treatment for a disease that is useful but can have side-effects- such as when we receive radiotherapy treatment to kill cancer cells but some of our non-cancer cells also get killed. Understanding how our immune system responds to such damage, and how it can malfunction to either fail to deal with infection or cause harmful effects to the damage, is crucial in designing new therapies that help our bodies protect itself from external threats.

What outputs do you think you will see at the end of this project?

Outputs will include:

1. A better understanding of how the immune system responds to infection with different types of pathogen

2. A better understanding of how radiation therapy affects the immune system in normal tissue to cause potential side effects

3. Publications that will inform the work of other scientists around the world

4. Development of potential novel targets for therapy for the benefit of the general public

5. Dissemination of understanding to the general public, for example via school visits and public engagement events

Who or what will benefit from these outputs, and how?

In the short- to mid-term (1-3 years), the research will aim to benefit other scientists in the field by providing important results that we can publish in scientific journals that inform the research of others.

In the mid- to long-term (3-5 years) the research will aim to benefit patients by providing new targets for disease therapies- for example, ways of treating patients to promote the immune response to infection, or ways of reducing the side-effects of radiation therapy in cancer patients. The eventual hope is that these findings will therefore be passed on to the general public in the form of new medicines.

How will you look to maximise the outputs of this work?

We aim to publish all research findings from these studies in peer-reviewed journals to inform the research of other scientists worldwide. We will aim to publish negative as well as positive data, to ensure that any approaches that do not result in positive results are not repeated by other scientists.

We will also publicise the findings of the research to a wider audience. For example, during the period of the last Home Office Licence, I took part in an event called 'Pint of Science', where I engaged members of the public in the pub about the specific research we do, including the importance of animal studies, and the potential impact that these studies have for human health.

Species and numbers of animals expected to be used

Mice: 10000 in protocol 1 (breeding protocol) 13000 in experimental protocols

PREDICTED HARMS

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use mice in our studies, as they have proven over many decades to be an incredibly valuable model of the human immune system. The human immune system is very complex, with interactions between different types of immune cells within different tissues determining the overall response to infection and other external stimuli. It is therefore unfortunately not possible to use isolated cells, or other lower organisms to model complex immune responses.

Specifically for this project, mouse models have proven extremely valuable in determining responses to infection with different pathogens, such as viruses, bacteria and parasites, as the responses to these pathogens mirror very well the responses seen in humans. Additionally, mice are proving an invaluable model to determine how our tissues respond to other external threats, such as radiation therapy that we study in our project. Radiation therapy is a critical treatment for cancer, but causes short- and longer-term side-effects, which are believed to be driven by the immune system. Mouse models of radiotherapy therefore allow us to dissect the involvement of complex immune interactions in causing such side-effects.

We will generally use adult mice in our experiments (>6-8 weeks of age), as at this age the mouse's immune system has developed to a degree that models the adult human immune system. However, important data suggest that modulation of the immune system in pregnancy and nursing can alter the susceptibility of offspring to external insults such as infection. Therefore, in some experiments we will treat pregnant and nursing mice with substances that alter their immune system, and then subsequently infect their offspring to try and uncover important ways in which alterations in the immune system during pregnancy and early life can have important effects on the immune system of offspring.

Typically, what will be done to an animal used in your project?

There are two main procedures that the animals will be subjected to during the project, which are both key external stimuli that alter our immune system and can have detrimental effects on our health: infection and radiotherapy. The ultimate aim is to determine ways in which to improve the outcome of infection and radiotherapy.

We will use a number of different infectious pathogens to determine important immune cells and molecules that regulate responses to infection. These are viruses, parasites and bacteria. These pathogens will be administered, in the majority of circumstances, via their natural infection route- either via the nasal passages for respiratory pathogens, or via the oral route for oral pathogens.

We will also re-infect mice after they have recovered from their first infection. Sometimes this will be with the same type of pathogen, to determine how so-called 'immunological memory' is formed- a vital cornerstone in how vaccinations work to keep us healthy. In other scenarios we will re-infect with a different type of pathogen, to see if the initial infection makes it more or less likely that the immune system deals well with the subsequent challenge.

In other separate experiments, we will treat mice with radiation, to mirror radiotherapy given to cancer patients. Although extremely important in helping treat cancer, radiotherapy can cause short-term and longer-term damage and side effects in normal tissue, which is believed to be caused by our immune system. Our experiments will involve giving mice a very targeted dose of radiotherapy to either their intestinal or lung region,

and attempt to determine how the immune system drives these short and long-term side effects.
In >95% of all experiments, we will analyse the tissues and cells of mice via post-mortem analysis. In
<5% of experiments, we will take blood samples during the experiment to monitor the immune system, before analysis of immune cells in tissues by post-mortem at the end of the experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

What happens to the mouse after infection will depend on the specific type of pathogen they have been infected with.

A single infection with viruses or intestinal bacteria can lead to transient inflammation and weight loss. Weight loss typically appears gradually during the first week of infection. At the peak of illness mice may show a hunched posture and reduced mobility, but in mice where the immune system is not altered in any way, 100% of mice are expected to recover.

A single infection with an intestinal parasite typically leads to no specific adverse effects being observed (e.g. infection with Trichuris muris). Infection with some other intestinal parasites (e.g. Trichniella spiralis, Toxoplasma gondii) typically leads to intestinal inflammation and weight loss, similar to viral or bacterial infection mentioned above. As above, in this case mice where the immune system is not altered in any way are all expected to recover.

We will also re-infect mice that have been previously infected, but only after full recovery from initial infection. The outcome of the mice will depend on which pathogen they are re-infected with; if this is the same pathogen that they were initial infected with, they will typically have generated so-called ‘immunological memory’, so their immune system will likely deal with the infection more quickly and efficiently, reducing symptoms seen. If the mice are infected with a different pathogen, our hypothesis is that, at least in some circumstances, this will cause an improvement in symptoms due to the body having adapted to the previous infection.

We will also subject some mice to either a single or fractionated dose of radiation, specifically targeted to the intestinal or lung region. Of note, we will not irradiate mice that have previously been infected.
This targeted radiotherapy (at the highest dose we will use) typically causes weight loss from days 3-4 post-treatment, peaking at days 5-7, before recovery to previous body weight by days 9-10 post- treatment. We will also monitor mice to determine longer-term side effects of radiotherapy, which may result in some further weight loss but this is typically very infrequent (<5% of mice).

In all of the above experiments, we will have some mice that are not infected or subjected to infection or radiotherapy as a control, which will not experience any weight loss or other symptoms. We will also have other mice that do receive infection or radiotherapy, but also treatment with control substances or substances that lead to modulation of the immune system. We estimate that approximately ~60-70% of mice used in the licence are expected to develop symptoms described above. Transient pain may occur on injection of

substances/cells, though in some instances they are anaesthetised when this procedure occurs.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

This project works to a maximum severity of moderate. With uninfected/untreated controls, and the use of therapeutics that aim to reduce symptoms of infection or radiotherapy, we estimate that ~60-70% of the mice undergoing these procedures will reach the moderate severity limit, with the remaining 30- 40% staying below this limit.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

External threats such as infection and radiotherapy can cause serious health issues in humans, and this is a result of complex interactions between many different types of cells in the body in complex organ systems. We know from our and others previous work that complex interactions between many different immune cells, and also the structural components of the organs, are vital in regulating the immune system. Thus, although it is possible to culture immune cells cultured in the laboratory (e.g. cell lines or cells obtained from mice or humans), such experiments do not take into account blood and air flow, the impact of the nervous system, nor interactions with non-immune cells in the context of complex organ systems. We will use cells and so-called organoids from mice (organoids being tissue isolated from mice and grown in the laboratory) to partially replace mice for pre-screening and hypothesis testing, but unfortunately there are no suitable full replacements for animal models currently.

Which non-animal alternatives did you consider for use in this project?

Models considered were:

Cell lines

Primary cells isolated from humans 3D organoid cultures
Primary human tissue

Why were they not suitable?

Cell lines: There are cell lines that were originally derived from different types of immune cells. However, these are often very much altered from the immune cell types found in vivo, and cannot model the complex interactions between different immune cells and non-immune cells in organs systems. These complex interactions are crucial in the overall outcome of infection and radiotherapy, which are required to determine whether specific treatments may improve outcome.

Primary cells isolated from humans: An improvement on the use of cell lines, in terms of cells reflecting the actual cells found in humans, are cells isolated from the blood or tissues from humans. However, this approach still comes with the issue that they cannot be used to mirror the complex interactions that are crucial in the overall outcome of infection and radiotherapy, and which are required to determine whether specific treatments may improve outcome.

3D organoid cultures: In recent years, models have been developed that use tissues from humans to produce a 3D structure, or so-called organoid, aimed at mirroring a human organ, and that can be seeded with different cells in an attempt to recapitulate the complex environment of an organ.
However, unfortunately such organoids are still limited in their complexity as only limited immune cells can be seeded into them and their make-up only models limited aspects of a real organ (e.g. no air or blood flow like a real lung, no movement of food and waste and no complex microbiome like a real intestine). Additionally, such organoids do not model the complex inter-play between organs that data has shown is extremely important to overall regulation of the immune system.

Primary human tissue: These tissues are very useful for working on human immune cells that have come from a complex environment. However, as with the above examples, major weaknesses include having to isolate cells and work on them outside their complex environment, and there is no ability to study the complex interplay between different organ systems and structures. An additional difficultly is the ability to obtain enough samples from individuals to build up a timeline of outcomes to infection/radiotherapy to interrogate mechanisms of action of the immune system. Obtaining such samples is not possible practically or ethically.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

There is an extensive literature using in vivo models of respiratory and intestinal pathogen infection, which we have used for over 10 years over two previous project licences. We have been carrying out radiotherapy models with collaborators for the past 4 years, with our collaborators having over 2 decades of experience using such models. Based on these experiences and working with expert biostatisticians, we have estimated numbers in all protocols based on using 4-6 mice per group, which have previously provided strong statistical power and mitigated the need for multiple repeats. Data collected in preliminary experiments will be used to compute the sample size needed in follow up studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

1) Sharing of mice for experiments: We are a large community of immunology-focussed researchers, many of whom use mouse models in their projects. There is overlap in some of the infection and radiotherapy models we all use, thus with correct timing and local communication, multiple scientists can obtain tissue and cells from mice undergoing a particular procedure. This has been ongoing during both of my previous project licences, and will continue in the current one to reduce the numbers used.

2) Confidence in reproducibility due to experience with models: During the past decade I have held a project licence, we have become experienced with knowing what variability and practical considerations are for specific models. For example, in some experiments (e.g radiotherapy experiments) the delivery of radiotherapy is machine-driven and very accurate both in targeting and dose. Therefore we generally use ~4 mice per group for these experiments knowing that variation is not a major issue. In some infection experiments, such as respiratory infections, delivery of the intranasal dose is somewhat more technically challenging, and variation is somewhat higher. Thus, we regularly use 5-6 mice per group here, knowing that any smaller groups would likely lead to non- significant results.

3) Use of available online resources: The NC3Rs provides extensive online resources to consider a Null (H0), or alternative (H1), hypothesis that enables the correct number of inter- and intra- experimental repeats to be calculated. For new experiments we will use these resources with the aim of reducing numbers used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As in our previous two project licences, we will reduce mice used by sharing tissues with other groups. For example, our local collaborators also employ models of lung viral infection, and our groups communicate to ensure that we share animals as much as possible. Similarly, other local collaborators employ intestinal parasite infection models and we regularly communicate with their team about potential for shared mouse use.

During the previous project licence, in consultation with the local animal staff, we have adopted procedures to more closely monitor breeding and make breeding more efficient (e.g. keep stock male and female cages that can then be bred if needed rather than constant ‘tick-over’ cages that produce litters continuously). We will continue these procedures going forward in this licence.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We only employ infection and radiotherapy models that have been refined over the years to minimise animal distress and replicate the human scenario better. Procedures involve administration of virus, bacteria and parasites typically by inhalation or oral gavage, targeted radiotherapy using the Small Animal Radiation Research Platform (SARRP) and injection of immune modulators aimed at reducing disease severity.

We constantly strive to reduce any animal suffering experienced. We closely monitor mouse condition (e.g. body weight, mobility, fur condition, body condition score) very regularly during procedures- specific monitoring depending on the specific procedure. At times where our previous experience have shown us that peak symptoms occur, we increase the frequency of monitoring. When anaesthetic is required for administration of substances (e.g. respiratory infection), we typically use inhalational, which allows recovery within a few minutes and prevents mice from losing body temperature whilst immobile.

Why can’t you use animals that are less sentient?

Unfortunately we cannot use less sentient animals for our experiments. Although animal such as Drosophila and zebrafish do have an immune system, this is a lot less complex than the mammalian immune system and so cannot respond in the same way to infectious and radiation challenges that we use in our project. Additionally, the pathogens used in the project- used to model closely human challenges- cannot be used to infect these less sentinel animals.

We cannot use terminally anaesthetised animals as we require the mice to develop immune responses to the external challenges, and to analyse the outcomes of these challenges over time and in response to therapies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

During the previous two project licences we have continuously refined procedures, mainly at the level of monitoring of mice to ensure minimisation of harm at all stages. In all experiments, if we detect any signs of unexpected symptoms we will increase monitoring to at least twice daily, and make a specific note about the experiment for future monitoring purposes.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow:

The government animal testing and research: guidance for the regulated community (https://www.gov.uk/guidance/research-and-testing-using-animals)

Morton et al 2001, Refining procedures for the administration of substances; Laboratory Animals, 35, 1-41

The NC3Rs webpage: https://www.nc3rs.org.uk/

Standard Operating Procedures developed with the animal facility and the named veterinary surgeon.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will ensure that all personnel who work under this project license routinely interact with the NC3Rs online resource (https://www.nc3rs.org.uk/). We will have a regular agenda item at group meetings to discuss ongoing animal experiments in the context of the 3Rs, and if there is anything that can be implemented based on recent advances.

195. Development of Novel Deubiquitinylating (Dub) Enzyme Inhibitors for Oncology

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Therapy, Deubiquitinylating enzyme, Protein degradation, Ubiquitin

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo
Rats	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We wish to develop new treatments for cancer by discovering drugs that inhibit a specific class of enzymes, termed DUBs (short for DeUBiquitinylating enzymes). We have developed molecules that can inhibit many of these enzymes and we aim to generate clinical candidates with the potential to stop tumour growth and progression. DUBs are implicated in several types of cancer including breast, colon and chronic myeloid leukaemia.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer remains a leading cause of mortality globally. This work will support the generation of new DUB inhibitor drugs that can uniquely target different types of cancer. Due to the

specificity that we are able to achieve, these new drugs will offer fewer side-effects than conventional treatments creating better quality-of-life for patients. Our new treatments may also be given in combination with existing treatments or novel treatments. In the process of developing the novel therapeutic approaches we will greatly advance DUB scientific knowledge, and are already active contributors to this field, publishing in high impact scientific journals and attending and contributing to scientific conferences.

What outputs do you think you will see at the end of this project?

This project will result in 4-5 candidate compounds to be developed as clinical drugs in 5 years. Each will a) be well tolerated b) demonstrate predictable circulating concentrations within the blood and c) show therapeutic potential in established models of disease.

Candidate compounds will be patent protected and developed outside the scope of this license to proceed to clinical trials either funded by the company or supported by partner organisations. Clinical trials will begin approximately 12 months after completion of studies under this license.

In addition, through building the understanding of how inhibition of DUB targets impact the disease progression, new information will be generated. It is anticipated that packages of work using studies with cells combined with experiments performed under this license will form the basis for further high impact publications.

Who or what will benefit from these outputs, and how?

In the short term, information generated and published at conferences or in journals will advance the field of DUB research and focus the field on the key DUBs involved in tumour biology.

In the long term, this research will lead to new efficacious and better tolerated treatments for cancer thus benefiting patients with potential economic benefit for companies marketing the novel drugs.

How will you look to maximise the outputs of this work?

We will collaborate with key researchers in the field (both academic and industrial) as well as charities supporting oncology research. It is expected that this will lead to publications and conference presentations on both successful and unsuccessful approaches. Through continual relationships with Key Opinion Leaders, active in Clinical Oncology we will have access to patient groups. Where there are relevant charities or patient groups for a particular cancer type we will approach them to share information and progress

Species and numbers of animals expected to be used

- Mice: 5760
- Rats: 580

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.
The majority of animals used in the project will be adult mice. Mice are the most appropriate species due to being widely used as an experimental model. The models that will be used are established in this species with efficacy translating to effective and safe treatments for cancer in humans. Adult animals will be used as the majority of cancers being treated will be in adult humans.

Typically, what will be done to an animal used in your project?

Typically, tumour cells will be injected under the animal's skin and tumour growth monitored by measurement, for example with calipers. Test compounds will be given to the animals, most likely through the mouth with a tube. Studies will either look at short term (under 24 hour) changes to the cells in the tumour after a single dose of the compound or longer term (one to four weeks) reduction in growth of the tumours with daily dosing of the compound. Animals will be maintained in groups in their normal cages throughout studies. About 5% of animals will have surgical implantation of tubes containing cells under their skin. These will then be administered compound for up to 8 days before animals are killed and the tubes removed for the cells to be grown in the laboratory. Approximately 1% of animals will receive irradiation either to allow the tumour to grow or to mimic radiotherapy given clinically. These animals will be dosed for up to 4 weeks with compound monitoring tumour growth as above. Where tumours cannot be measured with calipers, they may be imaged during which animals will be anaesthetised using an inhaled anaesthetic.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals will develop tumours at the site of creation. These will be monitored to ensure that they have minimal impact upon the animal, for example size controlled and no ulceration. The animal's weight gain will also be checked to ensure they do not experience weight loss. It is possible that the compounds administered have some side effects, such as causing transient nausea so animals will be observed to ensure these are mild. Irradiation can have an effect on the gut, causing nausea and irritation so animals will be monitored for weight loss or signs of discomfort/pain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity is expected to be mild in the majority of cases (95% of mice and rats). Moderate severity is possible in up to 5% of mice and rats.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?
A successful compound needs to have the characteristics to leave the stomach, enter the circulatory system and reach the tumour in order to block growth and cause shrinkage. In order the determine this, live animals are required as several body systems are working together to deliver the compound to the tumour. Whereas it is possible to grow tumour cells in culture, it is not possible to create the complex interaction between many different cell types that are involved in tumour biology in mammals.

In vitro (cells grown in the laboratory) assays will allow us to address important early-stage criteria such as enzyme potency, selectivity, off-target effects and to make predictions about the compound's metabolic stability before it is dosed in vivo (to animals). While the predictive capacity of these in vitro approaches is continually improving they cannot currently replace animal studies. This is because new drugs need to be tested in complex, biological systems with integrated functional pathways and this requires the use of animals. Sole reliance on in vitro biochemical and cell- based assays are not suitable to determine and identify key properties required of therapeutic agent in a complex biological system. Pharmacokinetics and biodistribution are examples of crucial endpoints that require integrated responses of the body to a drug that include components of absorption, distribution, metabolism and excretion. In vitro systems are getting better at predicting absorption and metabolism with Caco-2 cell flux and primary hepatocyte metabolic profiling, however they are not perfect. Moreover, distribution and excretion of a drug or its metabolites is impossible to accurately predict with in vitro methodologies. Indeed, it is recognised that co-ordinated use of both in vitro and in vivo allow robust translatable predictions to man that would not be achievable by in vitro or in vivo studies alone

In our specific case additional complications arise from the mechanism of action of our compounds as they are designed to act as reversible covalent inhibitors of DUB enzymes. This means that the compounds have the potential to bind to the target enzyme for longer than might be expected. The potential time disconnect between test compound concentration and initial effect cannot be replicated to sufficient detail in cellular or sub-cellular experiments without testing this in experiments involving the integrated system of a living animal. Moreover, potential covalent interactions with unidentified off- target proteins could result in physiological impact that would not be predicted from in vitro assessments.

Which non-animal alternatives did you consider for use in this project?

Our drug discovery programme uses many non-animal techniques such as cell-free biochemical assays, cell assays and computer modelling to address important early-stage criteria such as drug potency, selectivity and potential side-effects; metabolic activity is often initially assessed using liver or other tissue preparations (artificially, outside the animal). While these reduce the numbers of animals used they do have limitations and cannot (currently) fully replace animal models.

Before being tested in animals, the compounds will be profiled through experiments using human cells either growing in a single layer or forming three dimensional clusters similar to a tumour. This will form a large part of the project as a whole, with many compounds being tested and their progression stopped based on experiments using non-animal alternatives.

Why were they not suitable?

These models will contribute to the research but they cannot recreate the complexity of human physiology and re-create the digestive, circulatory and tumour environments working together.

Additional biological complexity is created in terms of our therapeutic mechanism of actions. The molecules that we produce bind to their target enzymes in a manner that is unlike the majority of drugs. Therefore, complex biological models are essential to adequately assess how they will behave, particularly with respect to how long they stay within a tissue relative to concentrations within the blood stream. Since the human DUB system shares much in common with that of other mammals, in terms of enzyme structure and distribution within the body, mice and rats can be effectively used as surrogates to show the impact of active drug levels in a biological system.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Mouse usage is split between support protocols and efficacy. Approximately 500 mice are estimated to be used in support protocols to allow breeding of GEMs (Genetically Engineered Mice). These will not transfer onto other protocols. Approximately 1500 GEMS will transfer onto efficacy protocols and as such have only been counted once in the total animal number.

Approximately 5250 mice and 580 rats will be used of efficacy protocols. This is split between four protocols -

Protocol	Number of Mice	Number of Rats
Dose finding and Tolerability	250	50
Hollow Fibre Assay	1000	150
Efficacy	3000	200
Pharmacokinetics and Pharmacodynamics (PK/PD)	1000	180

PK/PD Study Numbers are based on -

PK studies: 5 mouse and 1 rat studies per year, each using 9 animals

PD studies: 6 mouse and 1 rat per year, each using 30 animals (5 groups of 6 animals per study).

Dose finding and tolerability studies are based on 5 mouse studies per year using 10 animals and 1 rat study per year using 10 animals

Hollow Fibre Assay studies are based on 6 studies of 30 mice or one study of 30 rats per year

Efficacy studies are based on 10 mouse studies each containing 60 mice per year. This allows a group size of 10 in experiments with six experimental groups. Studies involving rats are likely to be less frequent, numbers are based on 2 studies of 20 animals per year.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The exact number of animals required varies between experiments but studies are designed to get the maximum amount of data from each animal, for example by testing more than one compound in a study with a single control group. Online tools such as the NC3R's Experimental Design Assistant have been used to further refine studies and provide information about experimental design, sample size and statistical analysis methods. Studies are designed to ensure that appropriate statistical power is achieved for each study through access to statistical expertise during planning.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

New protocols will be set up by running small pilot studies with smaller group sizes guided by literature and our own data. This will then be used to determine the minimum number of animals required for each study. Study design will ensure that the maximum information is generated from the minimum number of animals, for example by testing more than one compound in a study with a single control group or by using more than one cell type to create the tumours.

Measures to keep animal numbers to a minimum:

- Strict screening criteria with clear stop/go decisions to ensure only our best compounds progress to in vivo studies.
- Model choice - pharmacodynamic models requiring smaller group sizes will be used to select compounds for tumour models with higher animal numbers per group
- Adapting the study type - studies employing serial micro-sampling, reduced time-course, or cassette dosing (dosing more than one compound at the same time) will reduce animal numbers; consequently, full time-course studies will be performed downstream where fewer compounds are tested, reducing animal numbers further. Hollow microfibres will be implanted, each containing a different tumour cell type. This allows several tumour types to be assessed at the same time within the same animal.
- Avoiding duplication - if data is available elsewhere, some experiments may not be necessary; however, some may be replicated for validation purposes.
- Whenever possible, genetically modified animals will be obtained from researchers/suppliers to avoid re-creating these lines and to utilise their knowledge.

- Minimal numbers approach in tolerability studies with stepped dosing.

Experimental design principles - pilot studies and statistics:

- Each of the protocols described herein are established and standardised throughout the drug discovery field with well accepted animal numbers required for robust translational predictions. They have been run under previous or current licenses within the organisation.

- Pilot studies will be employed when working with compounds that have not undergone in vivo testing before. These will be in minimal numbers of animals using dose-escalation procedures to ensure animals are never unintentionally administered non-tolerated doses of a given drug.

- Sources of variation and bias will be reduced whenever possible through after-action reviews of studies to ensure future learnings. Moreover, advice will be sought from Establishment Licence Named Individuals, e.g. Named Veterinary Surgeon (NVS) and Named Animal Care and Welfare Officer (NACWO), so we are able to follow current best practice.

- Factorial experimental design and blocking will be applied whenever possible and animals randomised to treatment groups.

- Appropriate statistical methods will include independent t-tests and ANOVA on sequential and end-point data sets, regression analyses for time-courses and Log Rank tests for assessment of survival time (using surrogate endpoints). Frequently used models will be monitored for robustness and integrity (e.g. using trend plots).

- Routine statistical power analysis of conducted experiments will be undertaken to ensure that we are able to identify meaningful effects at 5% significance level with a power of 80%, and to adapt experimental design if our studies are over, or under powered. Separate power calculations may be necessary when considering experiments with male and female animals or both sexes together, and this will be taken into account.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The majority of models will involve injecting cells to produce localised tumours under the skin on the animal's side. The size of the tumour will be monitored to ensure it does not become large enough to affect the animal. In addition, bodyweight will be monitored. The level of pain, suffering, distress or lasting harm to the animal will be controlled by running

studies for the minimum length of time in which to see a drug effect. Measurement of tumour size is through non-invasive means causing minimal distress to the animal.

Where blood sampling is required, this will be via tail vein microsampling which is encouraged by the NC3Rs as it is minimally invasive, quick and limits blood loss. We have optimised methods to reduce the volume of blood that we need to a few microlitres to reduce the burden upon the animal.

In some cases the animals will be irradiated to allow the tumour to grow. In this case, the protocol will be optimised by limiting the length they are run for and the radiation dose given to cause the minimum pain, suffering, distress, or lasting harm to the animals.

Why can’t you use animals that are less sentient?

Immature animals are not appropriate due to the time it takes for tumours to grow and treatment effect to be determined. Similarly, these protocols cannot be done under terminal anaesthesia as this would only be suitable for very short term studies.

Zebra fish have been previously used to complement our research efforts but mouse better reflects the presence of our targets and their interactors. Mice and rats are the lowest species in which the (patho-) physiological function of DUBs in mammals can be studied and translated to the human physiology.

The physiology and architecture of mammals is required to accurately model the complex process of ubiquitinated protein processing.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Surgery: Non-surgical approaches will be utilised where possible. All recovery and long-term non- recovery surgery will be done aseptically (see the Appendix 4 HO Minimum Standards for Aseptic Surgery. See also other guidelines e.g. LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery http://www.lasa.co.uk/wp-content/uploads/2017/04/Aseptic-surgery-final.pdf

Anaesthesia and analgesia: studies involving surgical procedures will be conducted using suitable anaesthesia for the species and model with peri- and post-operative analgesia to best practice as advised by the NVS. Behavioural and facial patterns will be used to identify animals requiring additional pain relief.

When anaesthesia/analgesia are not required (e.g. dosing/sampling) we will use the least invasive route/method (e.g. in-dwelling cannulae or micro-sampling from superficial vessels); we will ensure that the volumes dosed/sampled will not be detrimental to the health of the animal.

Housing, husbandry and care: animals will be housed and cared for according to best practice. Licensees will be trained in recognising pain and suffering and abnormal changes in animal behaviour. Experiments will be supported by a well-trained and experienced centralised husbandry team. Surgery is performed aseptically under the standards outlined in the “Guiding Principles for preparing for and undertaking aseptic surgery” (LASA 2010).

Humane end-points: Animals will be checked closely for the onset of clinical signs and the frequency of these checks adjusted accordingly. Studies will be terminated at the earliest possible point to minimise adverse effects without affecting the science. Behavioural and facial patterns can be used to identify sick animals early.

We will use our knowledge and expertise (or that of other researchers) to predict and manage adverse effects; we will ensure that appropriate humane endpoints are developed, applied and refined as more information on the model becomes available.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the ARRIVE guidelines as recommended by the NC3Rs

For tumour studies, recommendations by Workman et al, British Journal of Cancer, 2010 will be adhered to, for instance to implement a maximal allowable tumour size.

Laboratory Animal Science Association (LASA) best practice guidelines will be adhered to for blood sampling.

The Experimental Design Assistant will be consulted when establishing a new experimental protocol and is available through the NC3Rs website.

There is a wealthy resource of guidance for best practice training and protocols available through the NC3Rs website, for example, handling and restraint, euthanasia, humane endpoints, welfare assessment, microsampling, anaesthesia, analgesia. Personal License (PIL) holders who are required to undertake any of these activities will be signposted to this website. Local establishment guidelines are in line by those endorsed by the NC3Rs and will be adhered to as well as incorporating refinements into our working practices. For any new procedures, the NC3Rs guidelines will be followed.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Attendance of NC3R-related seminars and monitoring the NC3Rs website. Staff working on the license will be encouraged to bring the 3Rs into their work and it will be part of their performance objectives to do so.

The establishment is a shared facility and the implementation of the 3Rs is encouraged throughout with periodic meetings sharing refinements to welfare and additionally interactions with the NVS, NACWO and Named Information Officer (NIO).

Attendance at conferences and formation of key collaborations to keep abreast of advances in non- animal alternatives, such as 3D modelling of tumours with human cells, organoids or tissue in more physiologically-relevant environments.

196. Studies on Lyssavirus Infections and Vaccine Protection Against Lyssaviruses

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words
Rabies, Lyssaviruses, Vaccine, Pathogenesis, transmission

Animal types	Life stages
Mice	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aims are to understand the different routes of lyssavirus infection and to assess the efficacy of vaccines and vaccine candidates in mouse models.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Rabies is a fatal disease caused by members of the genus Lyssavirus. Globally, over 59,000 people die of rabies each year. The currently available vaccines can protect against the rabies virus (a member of phylogroup I lyssaviruses) but require multiple doses to achieve the required level of protection. This makes it difficult to deliver vaccines in resource-limited countries. The existing vaccines are also less effective against other phylogroup I lyssaviruses and not effective against non- phylogroup I lyssaviruses. Therefore, more efficacious vaccines and vaccines with a broader coverage need to be developed.

Whilst for classical rabies, transmission occurs most readily through the bite of an infected dog transferring the virus through the saliva into the victim; however, the transmission between bats or from bats to other animals is poorly understood. Therefore, it is planned to assess the efficacy of different inoculation routes in mouse models (as a proxy to bats/cats) to determine likely routes of infection. This will help to assess the potential danger posed by a rabies bat. For example, dead bats may be eaten up by scavenging animals and cats often catch bats with their mouth; therefore, it is important to understand the effectiveness of the oral route of transmission.

Mouse models are needed to test the efficacy of candidate vaccines and the existing vaccines for post- exposure treatment. The mouse models will also be used to test the effectiveness of different routes of infection by lyssaviruses.

What outputs do you think you will see at the end of this project?

The project will increase our understanding of the routes of transmission of the lyssaviruses.

We want to develop more effective vaccines against rabies that require fewer doses, easier to transport without cold chain and are efficacious against divergent lyssaviruses.

All results will be published in high impact journals, presented at international conferences and communicated to policymakers and the public.

Who or what will benefit from these outputs, and how?

The information on the routes of infection will be used to educate the public on how to avoid the dangers posed by lyssaviruses.

In addition, it will be beneficial to have more immunogenic rabies vaccines, cost less, induce neutralising antibodies faster, protects against most or all lyssaviruses and are stable at the ambient temperature. These characteristics will make the deployment of vaccines easier, especially in resource-limited countries or areas where many people are still dying from rabies. It will contribute to the global strategic goal of Zero by 30, i.e., no human death caused by dog rabies by 2030.

How will you look to maximise the outputs of this work?

The information acquired will be published in scientific journals and disseminated through press releases, social media and the public engagement activities such as World Rabies Day. The unsuccessful approaches will be published in journals that accept negative results such as the Journal of Trial and Error. The advice on the following website will be

consulted regarding the publication of the null and neutral results: https://s-quest.bihealth.org/fiddle/.

Species and numbers of animals expected to be used

- Mice: Mice 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the biological system of choice for these studies. They are sufficiently immunologically similar to humans to allow confident extrapolation of findings, yet they are more amenable to laboratory experimentation than dogs, cats or non-human primates which would otherwise be the species of choice for these studies. Multiple mouse models have been well-established for assessing vaccine efficacy. Mice from 3 weeks old will be used as they are able to generate good levels of immune response to vaccination. The BALB/c mice will be the model of our choice as we have used them extensively in the recent past. Other mouse strains may be considered in situations that the BALB/c mice are not suitable, e.g., to enable the repeatability of the results in a collaborative research project or when the novel vaccine needs to be tested in a specific genetic background.

Typically, what will be done to an animal used in your project?

Mice will be vaccinated via intraperitoneal (IP), intramuscular (IM) or sub cutaneous (SC) routes. One or a maximum of 3 doses of vaccine may be administrated. A small amount of blood will be removed from a superficial vein to measure the immune response to vaccination. The amount of blood taken will be less than 10% of circulating blood volume being removed at one time and not more than three times and no more than 15% of the circulating blood volume over a 28-day period.

The animal may be challenged with a virus. Before the challenge, the animal will be anaesthetised (AB). If animals are challenged by the IC route, an analgesic will also be administered (AB). The virus may be administrated intramuscularly (IM), intranasally (IN), conjunctively (C) or orally (O). For peripheral challenge (e.g. IM/IN/C), the virus may be inoculated at two sites (e.g., different legs/nostrils/eyes) on the same day.

The animals will be kept until a humane endpoint is reached defined by the clinical scoring system or by the end of the experiment. Before termination, mice will be placed under terminal anaesthesia and exsanguinated by cardiac puncture. Mice will then be killed by a Schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals will suffer transient pain when needles are used to administrate vaccines or viruses. The animal is anaesthetised, and analgesia is given if intracerebral inoculation is undertaken,

If the animals are not protected by a successful vaccine candidate, when the virus multiplies in the body, usually after day 7 (but can be much longer) post-inoculation, the animal may display nonspecific early signs of infection, e.g., rough coat, hunched back, hyperactive or lethargic, and starting to lose weight. The animal will be euthanised if these signs persist or progress to the specific signs of rabies infection: incoordination, spasm and paralysis. Additional late evening clinical checks are introduced during the disease-onset period to minimise the duration of any suffering to hours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most animals are expected to be in either mild (>2/3) or moderate severities (<1/3) categories. In the past, less than 2% of animals died after the IC procedure and vaccination.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals are required in order to study the immune response to vaccination and to assess the protection afforded by vaccines. The pathogenicity of a virus can only be defined by infecting animals.

Which non-animal alternatives did you consider for use in this project?

No non-animal alternative is available to achieve the research goals currently. Cell cultures will be used to assess the growth of the viruses and the virus titre before infecting animals.

Why were they not suitable?

No non-animal alternative can display signs of infection or generate antibodies after vaccination or infection.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?
The number of animals required has been estimated based on our previous experience of working on different protocols described in this application. The number of viruses, vaccines, routes of inoculation and dosage levels for each protocol was first projected. The need for control and test groups is considered. The effect sizes were estimated and then the number of animals was calculated to ensure statistical and biological significance can be achieved.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Each experiment involving animals will be carefully designed to ensure the best possible chance of achieving the desired outcome. This will be through pilot studies, followed by confirmatory experiments designed to test specific hypotheses. Where appropriate, full power analyses (e.g. using nQuery Advisor or G-power) will be used to calculate sample size, taking into account the likely effect size.
Statistical input for studies will be sought from the biostatistician to avoid bias and to ensure validity of data. Where multiple possible outcomes are possible or a complex design is required, then the resource equation will be used as an alternative. Where potential multivalent vaccines are tested, control animals can act as controls for several vaccine candidates and as such animal numbers will be minimised. It is important to note that study designs will be coordinated with those of collaborators such that numbers of animals required are reduced and work is not duplicated unnecessarily.

The Experimental Design Assistant (https://www.nc3rs.org.uk/our-portfolio/experimental-design- assistant-eda) will be used in combination with local ethical review, including by a biostatistician, to ensure that the minimal required number of animals is used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Before designing the experiment, extensive literature research will be carried out to consider the information already available. Extensive in vitro testing will act as an initial screen, thereby minimizing the numbers of in-vivo experiments needed. Sometimes, pilot studies with a small number of animals may be necessary to define essential parameters before undertaking a major study. If possible, computer modelling will be explored to simulate the test in animals. Where possible without jeopardising the interpretation of results, in-bred animals will be used to minimize random variation and therefore reduce the sample size needed to detect the same signal. The maximum amount of information will be extracted from each experiment, i.e., animal tissues will be used to identify immunological markers or to be used as positive control materials for diagnostic tests.

All studies performed for research activities are carried out to ISO9001 quality standards. Our establishment is committed to complying with PREPARE and ARRIVE guidelines. Further, all studies are scrutinised by the AWERB onsite at our establishment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice represent a good choice for these studies as mouse models are well-established for studying lyssavirus vaccination and pathogenesis. Anaesthesia and analgesic will be used to eliminate/minimise pain. Environmental enrichment will be provided to enable mice exhibit natural behaviours. Mice will be handled by trained staff to minimise stress. Clinical scoring system will be used to determine the humane endpoint to reduce the length of any suffering. Heating mat may be used to aid the recovery after performing procedures needing anaesthesia. Recovery gel will be used as a nutritional support post-anaesthesia/inoculations if needed.

In addition, the vaccinations are carried out via intraperitoneal (IP) or intramuscular (IM) routes. Both IP and IM routes are difficult to perform properly and induce considerate discomfort to mice. If possible, the subcutaneous (SC) route of injection will be used as a refinement.

Blood sampling via saphenous veins will be considered instead of the tail veins as a refinement. For tail vein sampling, mice will be warmed for 10 minutes at no higher than body temperature (37°C) to dilate the blood vessels. A suitable restrain devise will be used and the tail will be cleaned with alcohol cellulose wipes. A lance or needle will be used to puncture the vein under good lighting that is suitable for both the operator and mouse.

Why can’t you use animals that are less sentient?

So far, no less sentient animal models exist for studying rabies infection or vaccination. Mice are the choice for these studies. They are sufficiently immunologically similar to humans to allow confident extrapolation of findings, yet they are more amenable to laboratory experimentation than dogs, cats or non-human primates which would otherwise be the species of choice for these studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The animals are acclimatised for at least 7 days before starting the experiment and checked at least once a day. The animals under study are routinely monitored twice a day or more if they are in the phase of rapid disease progression. Staff are trained to recognise the signs of suffering. Marker pens have now been used to mark animals so that to minimise the stress caused by ear-tagging. The cages are warmed after the IC procedure to aid the recovery. An analgesic is used for IC procedure and anaesthetic is used when necessary to minimise pain and distress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The following guidelines will be consulted:

The PREPARE guidelines

Standards for laboratory housing are defined in the Home Office Codes of Practice for the husbandry and care of animals

https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/662 364/Guidance_on_the_Operation_of_ASPA.pdf

EU directive 2010/63/EU
Procedures With Care: https://researchanimaltraining.com/article-categories/procedures-with-care/ Culture of care: https://norecopa.no/more-resources/culture-of-care LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf laboratory-animals-veterinary-association-guidance-to-named-veterinary-surgeons-april-2016%20(1).pdf

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Subscribe and read the NC3Rs newsletters. Attend NC3Rs events, workshops, webinars and refreshment training to keep abreast of 3Rs advances. Learn from colleagues and get advice from veterinary surgeons, NACWO, NIO, NTCO. Before each experiment, search literature and 3Rs websites (such as https://www.nc3rs.org.uk/3rs-resources, Koner, norecopa and linked websites) for any improvement of procedures published. The study protocol is scrutinised at the AWERB meeting for the methodology to be used and good suggestions incorporated into the study. At the end of the experiment, a washup meeting is held to discuss issues and improvements that can be made. A review meeting is held yearly with other groups performing animal experiments to discussed 3R issues and improvements can be made.

197. Mechanistic Immune, Inflammatory and Arthritis Models

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Inflammation, Autoimmunity, Arthritis, Therapy

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to aid the development of one or more novel therapeutics for the treatment of chronic inflammatory/autoimmune diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There is still a clinical unmet need in chronic inflammatory and autoimmune disease and this project aims to address this by helping to develop novel therapeutics that not only treat the symptoms but also halt the underlying disease process which has the potential to deliver longer term and more effective solutions to patients.

What outputs do you think you will see at the end of this project?
Overall this program of work aims to support the development of one or more novel therapeutics to treat patients living with debilitating autoimmune diseases such as rheumatoid arthritis.

Information gained from this program of work helps us to determine if a potential drug has an effect in a complex biological system. It also allows us to understand the dose and drug exposure required to see that effect. This information is used alongside in vitro human cell data to help select the most potent potential drug as well as to predict the dose we may need to use in clinical trials to see an effect in patients.

Where possible findings around our understanding of any novel biology will be published and shared with the wider scientific community at conferences.

Who or what will benefit from these outputs, and how?

The ultimate aim of this project is to benefit patients who are living with chronic autoimmune diseases. Due to the drug discovery process it can typically take up to 10 years from identifying a potential therapeutic target to developing a drug that can be prescribed to patients.

In the short-term, we expect to optimise in vivo models that can be incorporated into a rationale screening cascade. These models are then used to help us understand the effect of a potential drug on its target. They are also used to help us identify the most effective potential drug within a research project. This data is also used to aid our decision to progress a potential drug into the clinic or not. In addition, this work helps us to understand the biology of novel targets and the mechanism of action of novel therapeutics. It is anticipated that three or more research projects will benefit from the data generated from the in vivo models under this program of work.

How will you look to maximise the outputs of this work?

Where possible data on models as well as novel therapeutics is shared at conferences and published in the literature. We also collaborate with numerous other companies and Universities and share methods, protocols and best practises from our experience in this area of research.

Species and numbers of animals expected to be used

- Mice: 8,250
- Rats: 1,600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult rodents (mice and rats) will be used in this project as they have a mature immune system that shares many similarities with the human immune system. Rodents can

therefore be used to research novel pathways and support the development of novel therapeutics for potential human clinical use.

Typically, what will be done to an animal used in your project?

Animals may be treated with novel therapeutics which are typically administered orally or by injection. Animals will undergo additional procedures that will involve injections that give rise to an inflammatory response or the development of disease symptoms, such as swollen paws. The period of time that animals experience this will be kept to a minimum to generate the meaningful scientific data required. Studies under this project may last from just a few hours through to over a month. In all studies animals will be carefully monitored to ensure any discomfort they experience is limited as much as possible.

What are the expected impacts and/or adverse effects for the animals during your project?

To induce an inflammatory response or disease symptoms animals will receive an injection. Induction of an inflammatory response may give rise to swelling at the site of the injection e.g. ear swelling that can be measured. These short-term inflammatory responses typically last from just a few hours up to five days. In longer term models where we induce signs of arthritis, animals will develop inflamed paws in one or more of their paws. This will result in sore joints and animals may be reluctant to move. In

these circumstances we supply food on the cage floor to help support them to feed. In these arthritic models mice typically experience inflammed paws for up to twenty days. In these arthritic models mice

The period of time that animals experience an inflammatory response or disease symptoms will be kept to a minimum to generate the meaningful scientific data required. It is also anticipated that a proportion of animals that are treated with novel therapeutics will experience a more mild inflammatory response or a reduction in disease symptoms.

Through our experience in the types of in vivo studies run under this project we have implemented a number of refinements that benefit animal welfare. These include increased environmental enrichment, refinement in techniques, reduced length of time animals have disease symptoms and even assessing novel therapeutics in the pre-arthritic phase of the models where animals do not develop inflamed paws.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The maximum severity that an animal will experience in a study under this project is moderate. It is however anticipated that not all animals will experience this due to the type of inflammatory response and disease duration that can be used. Overall, it is expected that about 20% of both mice and rats will experience a mild severity and the other 80% will experience a moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Inflammatory, immune and disease responses in humans are complex and involve interactions between tissues and various cell types. This complexity cannot be completely mimicked in vitro hence the need to assess the effect of new medicines in animal models.

Which non-animal alternatives did you consider for use in this project?

Wherever possible, in vitro cell based assays will be used to study functional responses of immune cells in isolation or combination before the use of an in vivo model. An up-to-date knowledge of the literature and latest technology will help replace in vivo experimentation wherever possible.

This includes the following databases for alternative methods:

The John Hopkins Centre for Alternatives to Animal Testing (http://altweb.jhsph.edu)
Animal Welfare Information Centre (https://www.nal.usda.gov/programs/awic)
European Centre for the Validation of Alternative Methods (https://joint-research-centre.ec.europa.eu/eu-reference-laboratory-alternatives-animal-testing_en)

Fund for the Replacement of Animals in Medical Experiments, FRAME (http://www.frame.org.uk/)

Why were they not suitable?

At present no in vitro assay or computer model can fully replicate the complex interplay of cells, inflammatory mediators and cell-cell contact that occurs in vivo in animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are based upon the typical n numbers required in an individual experiment, the number of experiments likely to be performed over a 5 year period and the anticipated number of projects that will be supported. These numbers represent the maximum that will be used but it is anticipated that much fewer will actually be used. This will be dependent on the number of internal drug discovery programmes that are support over the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Optimum group sizes have been established, based on previous experience with these protocols taking into account experimental variability and level of disease incidence to ensure that scientifically meaningful data is generated. We also have access to experts in statistics who help us assess the number of animals that are required in our experiments. This helps ensure we use the correct number of animals to generate data. Our statistics group also helps us ensure we use appropriate statistical tests when analysing data.

We are also investigating ways of reducing the number of animals used overall by avoiding the unnecessary use of more than one set of controls and/or reducing the number of animals within control groups to a minimum by using historical data where appropriate.

Group sizes are constantly reviewed to ensure the minimum number of animals are used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To ensure the fewest number of animals are used, only the most effective novel therapeutics that have been pre-screened for activity in vitro will be examined in animals. An initial pilot study consisting of a single high dose group plus appropriate controls may be conducted initially to determine efficacy of a novel therapeutic prior to larger multi dose group studies. This will ultimately help to reduce or refine the groups required in a larger study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project uses short term models of inflammation and the immune response as well as models of arthritis. These models mimic processes that are found in human chronic inflammatory and autoimmune diseases such as rheumatoid arthritis. A number of the animal models in this project are well established both in-house and within the literature. However, no single model accurately reflects human disease and it is therefore necessary to study different models that model different components of human disease.

All procedures have been ethically reviewed and all animals undergoing procedures are monitored closely by trained staff that work closely with a veterinary surgeon. In addition, welfare scoring sheets are used to monitor disease severity and employ humane endpoints to limit suffering. These are under constant review and will be amended as appropriate.

Why can’t you use animals that are less sentient?

Rodents (mice and rats) are the most appropriate mammalian species for the models in this project. The generation of a robust inflammatory or immune response requires a

mature immune system, so adult animals are used. These responses and the development of disease symptoms can take weeks or months to fully develop and as such it is not possible to conduct studies under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We aim to limit the animal suffering through use of welfare scoring sheets. Humane endpoints are employed to limit suffering and disease burden and are constantly under review. When required increased monitoring is undertaken to minimise any impact on animal welfare.

In models where animals develop arthritis additional food, often in the form of wet mash, is placed in the cages at floor level to ensure ease of access which limits the stress on their swollen paws.

All cages are environmentally enriched for better animal welfare and we employ tunnel handling techniques to limit stress to the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidance will be taken from organisations such as the NC3Rs and LASA as well as the ARRIVE (https://arriveguidelines.org/)and PREPARE (https://norecopa.no/prepare) guidelines.

Specific guidance on the refinement of animal models of arthritis (Hawkins et al., 2015. Applying refinement to the use of mice and rats in rheumatoid arthritis research. Inflammopharmacol. 23:131-
150) has also been taken into account.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

There are many 3Rs resources available which include internal sources such as the animal welfare and ethical review board (AWERB) and the named information officer. Information on the 3Rs can also be obtained from external sources such as conferences or webinars. There are also outside organisations such as NC3Rs, norecopa and LASA (both website and conference attendance). We also seek advise from the named veterinary surgeon (NVS) who provides continuous professional development seminars on a range of topics. In addition, we take into account and implement published guidance that is directly relevant to the models in this project such as those from a working group on refining animal models of arthritis (Hawkins et al., 2015. Applying refinement to the use of mice and rats in rheumatoid arthritis research. Inflammopharmacol. 23:131-150).

198. The Impacts of Feed Materials and Functional Feed Additives on the Health and Welfare of Fish

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Enteritis, Inflammation, probiotic, prebiotic, soybean meal

Animal types	Life stages
Zebra fish (Danio rerio	adult
Rainbow trout	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to provide essential information surrounding the use of in-feed strategies to help reduce stress and disease encountered during the farming of fish. This research will enable improved feed formulations which will enhance the welfare of farmed fish, as well as increasing farm productivity and underpinning food security.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.
Why is it important to undertake this work?

Aquaculture is the farming of organisms in the aquatic environment. Half of the world's seafood now comes from aquaculture, and this is expected to increase to 60% by 2030, and will increase further beyond this date. Fish farming is therefore an increasingly important contributor to global food security. At any given time, there are over 100 billion fish being reared in aquatic farm environments. It is important to ensure that these animals are farmed productively, but also in a way that supports good welfare.

Plant feedstuffs are increasingly used in fish diets to increase the sustainability of commercial fish feeds. However, intestinal disorders in fish fed high levels of plant feedstuffs leads to reduced fish welfare, slower growth performance, poorer feed conversion and increased susceptibility to infectious diseases. It is therefore important to develop in-feed solutions that can prevent intestinal disorders and improve fish welfare, fish farm productivity and global food security.

In recent years the concept and application of functional health ingredients, such as immuno- modulants, prebiotics and probiotics, has received much interest as a preventive health management strategy across livestock production, including fish farming. This project will enable feed manufacturers to produce better fish diets that will have beneficial impacts on farmed fish health, welfare and productivity. The project will use an established and robust zebrafish model for the rapid assessment of functional health ingredients, as well as trials in key food fish species (salmon, trout, tilapia and catfish) to better understand the efficacy (and modes of action) of functional feed additives to alleviate intestinal inflammation, stress and dysbiosis.

What outputs do you think you will see at the end of this project?

The short-term goals of this project will include better understanding of:

1. The role of functional feed additives to reduce the negative effects of chemically induced intestinal inflammation in a model species.

2. The role of functional feed additives to alleviate dietary induced stress in food fish species.

3. The role of functional feed additives to alleviate stress induced by short-term feed deprivation, which is a common fish farm production practice.

Primary publications in scientific journals will be generated to inform the academic community of our findings, and commercial stakeholders and the general public will be informed through multiple other channels (e.g. social media, conference communications, publications in trade journals etc).

The long-term benefit of this project will provide important information to further the scientific understanding of the efficacy and mode of action of functional feed additives, which will be important to the academic and commercial sector on four levels:

1. To improve the decision pathway process and fast-track the implementation of dietary in-feed solutions to offset the use of environmentally polluting chemicals/antibiotics to combat disease.

2. To better inform the use of functional feed additives as a long-term sustainable approach to improving the health and welfare of farmed fish.

3. To improve the welfare and health of farmed fish.

4. To improve global food security by enhancing productivity and sustainability of fish farming. This is especially important as aquafeeds continue to be formulated with higher inclusion levels of plant-based protein sources, to replace less sustainable fish-based proteins from wild capture fisheries (i.e. fishmeal).

Who or what will benefit from these outputs, and how?

This research will have positive socio-economic, environmental and fish welfare impacts through improved feed formulations fed to farmed fish. The novel application of an early-stage whole-animal model will lead to the discovery of new feed additives that will help to deliver solutions for fish health and welfare applications. With increasing societal awareness of animal welfare and sustainable practice and the desire to minimise medicinal or chemical interventions, the present study offers innovative methods for improving welfare in an eco-friendly way, which should be acceptable to consumers and carry significant value for the agricultural/aquaculture industry. Ultimately, this leads to higher animal farming productivity, improved food security and better animal welfare, supporting United Nations Sustainable Development Goals 2 (zero hunger), 3 (good health and well-being), 12 (responsible consumption and production), 13 (climate action) and 14 (life below water). Beneficiaries therefore include farmed fish through improved feed formulations, the environment and general public through reduced environmental waste, fish farmers and fish consumers via improved productivity.

In addition, attaining a deeper understanding of the underlying mechanisms of gut health and how functional feed additives can help mitigate stress and improve the homeostatic mechanics will benefit

ichthyologists and other researchers working in this field as well as aquafeed and feed additive companies.

How will you look to maximise the outputs of this work?

Dissemination of information will be important to streamline the decision making process for use of successful feed additive candidates in future trials and expedite the use of more efficacious products in aquaculture production. The information provided by this project will lead to a better understanding of the mode of action of feed additives on fish immune responses and microbiome.

This will help provide data for our industrial partners, funding bodies and stakeholders (aquafeed companies; producers of feed materials and feed additives) to promote better health solutions to combat ongoing disease issues and develop better health management strategies for terrestrial and aquatic farmed animals. This long-term partnership with multiple stakeholders (>10 years) has to date ensured that >10 million fish per annum benefit from improved feed formulations and elevated welfare status. The present research programme will allow this benefit to extend further, ensuring that millions more farmed fish will benefit from further improved formulations.

Furthermore, this research project aims to produce at least three publications in scientific peer reviewed journals and dissemination at flagship conferences to ensure that scientific impact is achieved.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 3,000
- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Within the aquaculture industry there are at least 350 different finfish species that are farmed commercially around the world. These species have widely diverse physiology, gut anatomy, microbiomes, rearing conditions, diets, and nutritional requirements. These factors affect efficacy of functional feed additives and modified feed formulations. As such, proof of efficacy in one species does not guarantee efficacy in another. Therefore, studies are required in a range of target species, using relevant diets and feeding regimes (inc. feed deprivation). Four fish species have been carefully selected: rainbow trout, Atlantic salmon, tilapia and catfish. These species, which are widely used in nutritional and microbiological studies, will be used for the following reasons:

1. They are commercially important aquaculture species contributing significantly to food security (collectively contributing ca. 10 million metric tonnes per annum).

2. Collectively, their biological, nutritional and digestive physiologies cover a wide scope, meaning that they are a good model panel for the species used in the wider aquaculture industry.

3. There is a large body of literature from previous studies, which means a] many refinements to the protocols are already made which means that fewer pilot studies will be needed (reducing the numbers of animals to be used), and b] allowing for scientific comparison.

4. Their relatively large size allows sufficient biological material to be sampled.

5. They are of high interest to key stakeholders, including our funders.

The zebrafish will also be used as an early stage fish model, to better understand the mechanisms of action and to help fill current knowledge gaps of this pathology. The main reasons for using this species includes:

1. Fully characterized genome and proteome for molecular studies

2. A colitis pathology model has previously been developed and published in this species. This model will be adapted to model enteritis in the current programme of research. This obviates having to use more animals to develop a model in a different species.

3. Well characterised zebrafish stocks are available in-house, which will allow for highly precise and reproducible studies.

4. The species is widely used in scientific studies and thus the enteritis model we employ could be widely used by other research groups.

Typically, what will be done to an animal used in your project?

Protocol 1:

A typical fish in this protocol will be fed control diets or diets that include functional feed additives for up to 6 weeks in order to modulate their intestine immune responses and microbiome. Subsequently, intestinal inflammation will then be generated by intrarectal exposure to TNBS. Fish will be monitored to ensure recovery and will be returned home tanks and fed their respective diets (control or experimental) for up to 3 days. Fish will then be killed humanely and post mortem samples will be taken for downstream analyses of key immunological, microbiological and inflammatory biomarkers.

Protocol 2:

A typical fish will be fed diets containing moderate to high (species-specific) levels of soybean meal in diets supplemented with (treatment groups) or without (control groups) functional feed additives. This feeding regime will last for up to 10 weeks. Fish will then be killed humanely and post mortem samples will be taken for downstream analyses of key immunological, microbiological and inflammatory biomarkers.

Protocol 3:

Fish will be fed high quality diets (which do not induce intestinal inflammation) that are either supplemented with (treatment groups) or without (control groups) functional feed additives for up to 10 weeks. At this point some fish will be humanely killed and sampled post mortem, and others will then be deprived of feed for up to 7 days, which mimics periods of feed deprivation in commercial farming practices. After the feed deprivation period, some fish will be humanely killed and sampled post mortem, and others may be re-fed their respective diets (i.e. control or treatment functional feed additives) for up to 4 weeks. After this, fish will be humanely killed and samples taken post mortem for downstream analyses of key immunological, microbiological and inflammatory biomarkers.

The protocols are rated at a moderate level of severity. No fish will be undergo multiple procedures, so there will be no cumulative effects.

What are the expected impacts and/or adverse effects for the animals during your project?

Fish will develop an intestinal inflammation response (Protocols 1 and 2) or be feed deprived for up to 7 days (Protocol 3) which may cause some temporal discomfort (Protocols 1 and 2) or hunger (Protocol 3). Although unlikely, fish could display adverse effects including abnormal swimming behaviour (all Protocols), reduction of appetite (Protocols 1 and 2) or signs of hunger (Protocol 3).
These effects will be carefully monitored using a scoring index as part of the procedure to detect any changes. Fish will be monitored at least 3 times per day to check for development of any clinical signs. It is expected that most of these symptoms will be

transient, for example, the expected timeline for intestinal inflammation induced by TNBS should have peaked at 6 hours post injection and then inflammation will start to reduce rapidly with a full recovery within 72 hours. However, if clinical signs of welfare deterioration such as negative changes to animal behavior, loss of appetite or external signs of stress are observed and persist for more than 24 hours, the fish will be humanely killed so suffering does not exceed moderate actual severity.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Protocol 1:

Up to 40% of the fish will experience moderate severity. The remainder of the fish will not be exposed to the aspects of the protocol that will cause potential harms (e.g. they will be control fish).

Protocols 2 and 3:

Up to 85% of the fish will experience up to moderate severity (anticipated to be 50% mild and 50% moderate). The remainder of the fish will not be exposed to the aspects of the protocol that will cause potential harms (e.g. control groups that will not be feed deprived).

What will happen to animals at the end of this project?

- Killed
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our aim is to understand the effects of functional feed applications in finfish aquaculture. In order to model the different stressors that the fish may experience during the aquaculture production cycle we will need to use in vivo subjects to investigate the mode of action of specific test candidate functional feed additives. The specific aims of each objective within the scope of the project dictate that we require a whole fish system approach to understand how each test ingredient interacts with the mucosal lymphoid associated tissue of fish. Moreover, investigating the effects of each ingredient on the microbial ecology of the fish intestine under different environmental conditions will require whole fish as a working model to investigate these responses.

Which non-animal alternatives did you consider for use in this project?

As a vertebrate alternative, we have considered the use of in vitro gut cell cultures systems, as well as

ex vivo gut sac techniques.

Why were they not suitable?

The ex vivo gut sac model (i.e. partial use of an animal) and in vitro gut cell culture techniques (i.e. not using animals at all) are useful to investigate a specific responses of cells and tissues to specific exposure regimes. For example, our group uses the ex vivo gut sac model to study the effects of probiotics on the intestines of fish. These approaches have been incorporated in early stages of the research programme as part of a tiered approach. However:

1] understanding the complex relationships between the different microbial populations in the intestine of fish using the ex vivo model would not provide a true representation of this complex environment and bias the data by providing potential for false positive or false negative results. Part of the scope of this project is to model inflammation in the intestine to understand the mechanisms and dynamic nature of the fish gut associated lymphoid tissue (GALT) responses to different functional feed additives under different environmental conditions. The immune response of GALT is a complex milieu of innate and adaptive cellular and molecular responses that would not be possible to replicate using in vitro techniques.

2] the impacts of these changes extend to growth performance and feed utilisation, which cannot be assessed without the use of the whole organism.

For these reasons, it is essential to use the fish species.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The experimental design is for each treatment to occur in triplicate as a minimum, with the tank as the recognized replicate. Triplicated experimental design has been scrutinized in peer review and is well established for experimental work with fish. To determine the total number of fish needed in an experiment Power analysis has been used.

The numbers are protocol, species and assay dependent. For assessment of all objective aims a suite analytical approaches will be used. Typically, a minimum of 9 fish per treatment will be required for sampling. In a triplicated tank experimental design, this means 3 fish per tank. Where possible, all samples will be taken from the same fish to reduce the total number of animals required. However, some samples cannot be obtained from the same individuals (e.g. intestinal biopsies cannot be obtained from fish that are required for whole body analysis, such as carcass composition) so additional fish are required. In a study whereby sampling is scheduled at mid-point and end-point, a minimum of 15 fish per tank would be sampled.

However, to ensure a level of redundancy, to ensure good animal welfare (i.e. appropriate stocking densities – which are species-specific) and to ensure that our data are of relevance to the aquaculture industry (i.e. appropriate stocking densities) the overall number of fish per tank in some studies would need to be greater than 15. It is anticipated that between 15-40 fish per tank (depending on the species and Protocol) will be required when factoring in optimal stocking density to allow normal behaviour and good welfare. Fewer fish will always be used if and where possible.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our experimental design is informed by prior training (e.g. BSc, PhD, Home Office approved licensee training) and experience with working with the species and analytical approaches to be used.To achieve statistical power and attain meaningful results from each experiment proposed during this project power analysis has been used. For most quantitative experiments samples sizes will be set using power analysis generally using a significance level of 5%, a power of 80%, and a least practicable difference between groups of 25%. In terms of numbers of animals required, we expect 9 fish to be sampled in total per treatment (n = 3/tank/treatment per timepoint). The end points for each experiment under objectives 1 - 3 are two-fold: A cellular and molecular approach will be performed. Where possible, multiple sampling can be achieved using the same fish to reduce the number of animals required for each sampling. Molecular analysis of the microbiota will be performed to understand the effects on the microbial ecology of the intestinal tract. We have a number of established protocols for sampling that have been well established for over 15 years and have been scrutinised by scientific peer review.

To account for biological variation and for intra-tank variation fish will be randomly assigned to an experimental group. Where possible a factorial design will be used to minimise animals required to achieve scientific significance. To reduce technical variation for sample collection and analysis specific protocols will be followed to remove bias and enable studies to be published according to the ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In addition to good experimental design, our approach is underpinned by using appropriate stocking densities to ensure good welfare as well as a need for redundancy in the trials. These numbers are based on our long-term experience with these animals and real-word aquaculture stocking levels. Our experiments are designed to answer multiple research questions, thus reducing the overall number of experiments that need to be conducted, and reducing the number of fish used. In addition, samples will be made available and shared with colleagues and other researchers to ensure maximum scientific value is obtained from the animals to be used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We propose to use the zebrafish model to develop an in-vivo screening approach to discriminate the efficacy of functional feed additives to elicit a positive host immune response (Objective 1). Zebrafish will be exposed to sub-lethal concentrations of an inflammatory inducing agent (TNBS). The exposure concentration and duration of TNBS is calculated to minimise the chance of pain, suffering, distress or lasting harm. According to published literature, the dose being used is the minimum required to induce a “mild” inflammatory response of the intestine. The inflammation will subside after peaking at 6 hours, and thus there will be no lasting harm. Fish will be monitored 3 times a day using a species-specific welfare scoring index generated to identify signs of abnormal swimming orientation, lesions, scoliosis, scale and pigmentation loss. If clinical signs such as changes to animal welfare exceed those of the welfare scoring system, or persist for greater than 24 hours, the fish will be humanely killed so suffering does not exceed moderate actual severity. The specific zebrafish stocks used are mycobacteriosis- free.

In addition, rainbow trout (Objective 2 and 3), Atlantic salmon (Objectives 2 and 3), catfish (Objective
2) and Nile tilapia (Objective 2) will be used in this project. Rainbow trout and Atlantic salmon are well- established aquaculture organisms (with 3.3 million metric tonnes produced globally, worth more than
$20 billion), and comprise the majority of the UK aquaculture output in both value ($1.3 billion) and volume (180,000 tonnes). As carnivores, they are also highly sensitive to enteritis induced by plant rich

diets. Likewise, tilapia (with production of ca. 5 million metric tonnes), catfish (ca. 2 million metric tonnes) are important aquaculture species globally, and are fed diets containing very high levels of plant feedstuffs. Despite their omnivorous nature, studies have revealed that they too can suffer from enteritis. These are therefore highly relevant organisms to study, with billions of individual fish of these species that could benefit each year from improved welfare in aquaculture practices as a result of this research programme.

Besides the economic importance of the above aquaculture species, there is a requirement to understand the mode of action of the candidate functional feed additives in fish species with different feeding requirements and morphological and physiological differences in the digestive tract. To fully validate and characterise the effects of each candidate feed additive it is necessary to investigate each candidate when exposed to the different extrinsic environmental conditions such as temperature and intrinsic conditions (gastric vs agastric species; fish with and without pyloric caeca etc) across the different species. All of these factors will affect the efficacy of the functional feed additives to alleviate intestinal inflammation and thus proof of efficacy in these target food fish species is essential.

The levels of soybean meal used in these studies (Protocol 2) is determined based on species specific studies published in the peer-reviewed scientific articles which demonstrate the minimum levels required to induce intestinal inflammation. Our approach will produce a localised intestinal inflammation that is required for the study, but not one that is excessive, systemic or will cause whole animal welfare issues.

The duration of feed deprivation used in Protocol 3 for salmonids is considerably less (7 days) than these species may endure in the wild (e.g. over winter or during migration) and less than that which is sometimes employed in the farming industry. Salmonids are highly adaptable and recent studies published in peer-reviewed scientific articles have demonstrated that feed deprivation for salmonids for up to 8 weeks does not reduce welfare, and that fish can make a full recovery subsequently when feeding is returned. A 7 day deprivation is therefore unlikely to cause pain, suffering or distress, and will not cause lasting harm.

Why can't you use animals that are less sentient?

We will be using juvenile or adult fish for all experimental work as the specific aims of each objective within the scope of the project dictate that we require a whole fish system approach to understand how each test ingredient interacts with the mucosal lymphoid associated tissue (MALT) of fish, it's microbial ecology and how this alleviates inflammation and impacts overall fish growth performance and health status. We cannot use earlier life stages (e.g. first feeding organisms such as fry) because the immune system, microbiota and digestive tract will not yet be fully developed. In addition, such small sized individuals would not be able to provide the relevant volume of biological material required for our analyses (and would lead to higher numbers of fish needed to be used as a requirement to pool material from numerous individuals). Using fry would also not be possible due to the requirement to work on whole blood for haemato-immunological assays, which could not be adequately sourced from fry.

In less sentient beings, the establishment of both the intestinal microbiota and the cellular and molecular composition of the immune responses of MALT of fish would not replicate the complex species-dependent interactions of these components.

Therefore, we cannot use less sentient organisms such as invertebrates.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Zebrafish (Protocol 1) will be exposed to low concentrations of an inflammatory inducing agent (TNBS). The exposure concentration and duration of TNBS is calculated to minimise the chance of pain, suffering, distress or lasting harm. According to published literature, the dose being used is the minimum required to induce a mild inflammatory response of the intestine. The inflammatory response itself is mild, but it will persist for up to 72 hours (having peaked at 6 hours, and then subsiding after) and so we rate the procedure as moderate.

The levels of soybean meal used in these studies (Protocol 2) is based on species specific studies published in the literature, which demonstrate the minimum levels required to induce intestinal inflammation. We have selected levels that will induce only a localised inflammatory response, and is sufficiently low to avoid triggering a systemic whole organism inflammatory response or fatalities.

The duration of feed deprivation used in Protocol 3 for salmonids is considerably less (7 days) than these species endure in the wild (i.e. over winter) and less than that which is sometimes employed in the farming industry. Salmonids are highly adaptable and recent studies published in peer-reviewed journals have demonstrated that feed deprivation for salmonids for up to 8 weeks does not reduce welfare. A 7 day deprivation is unlikely to cause pain, suffering or distress, and will not cause lasting harm.

Fish will be monitored at least 3 times a day using a species-specific welfare scoring index generated to identify signs of abnormal swimming orientation, lesions, scoliosis, scale and pigmentation loss.
Increased frequency of monitoring will be employed if signs emerge. If changes to animal welfare observations exceed those of the welfare scoring system, the fish will be humanely killed so suffering does not exceed the relevant severity threshold

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will be exposing the fish to sub-lethal levels of stressors. All protocols are well refined based on the published literature that demonstrates the sensitivity of zebrafish to the inflammatory inducing agents (TNBS or soybean meal). The literature shows the levels required from low (localised inflammation) all the way to high (inducing systemic inflammation and mortality). We have selected the minimum doses required to induce a low level of localised intestinal inflammation, which is sub-lethal. We will follow these protocols as well as relevant documents published by the NC3Rs, as well as consulting the University Animal Welfare Ethical Review Board and the Named Veterinary Surgeon.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

This will be approached by:

1. Regular checks on the NC3R's website.

2. Contact with the Named Information Officer for any current updates.

3. Engagement with AWERB

4. Frequent checking of scientific material relating to fish welfare and biostatistics in scientific articles, documentation from relevant NGO's, social media, general media and other relevant publications to ensure that we maintain best practice

199. Environmental and Placental Contributions to Pregnancy and Life-Long Health

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Fetus, Placenta, Fetus and maternal Health, Developmental Programming, Therapeutic interventions

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To determine the impact of the environment of the mother and father on pregnancy outcomes (Aim 1), life-long health of the child (Aim 2), and the health of the mother after birth (Aim 3). To determine if interventions given to the mother, father or both improve pregnancy outcomes and life-long health (Aim 4).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In developed countries like the UK, more than 17% of women develop complications during pregnancy. Such complications include low birth weight, development of diabetes in pregnancy (gestational diabetes), development of high blood pressure in pregnancy (pre-eclampsia) and premature birth.
These complications can threaten the life and health of the mother and developing child. They also have life-long impacts on the health and wellbeing of the mother and her child. Women who develop diabetes or high blood pressure during pregnancy are more likely to develop diabetes and heart disease after birth. Babies with low birth weight are at greater risk of developing obesity, diabetes, heart disease and neurological impairments as they grow older. Pregnancy complications and their related life-long impacts are major emotional and financial drains on societies worldwide. Certain genetic factors and environmental conditions (like undernutrition, obesity, and stress) increase the risk of pregnancy complications. However despite this, we lack information on the pathways by which this may occur. The placenta is an essential organ that develops during pregnancy. It is responsible for transporting all the nutrients and oxygen a fetus needs to grow and develop. The placenta also produces chemical messengers that signal to the mother to support fetal growth and development.
This programme of work will determine the importance of the mother's and father's environment for growth of the fetus and the placenta, as well as health of the mother during pregnancy (pregnancy outcomes; Aim 1). It will also follow the health of the child and mother after birth to see if there are long-lasting impacts (Aims 2 and 3). It will also identify interventions and treatments given to the mother, father or both parents to improve pregnancy outcomes and the life-long health and wellbeing of the mother and child (Aim 4). This work is important as there are no current treatments available to prevent the development of pregnancy complications or their related life-long health risks.

What outputs do you think you will see at the end of this project?

The outputs of this programme of work will be information on the impact of the environment of the mother, father, or both parents in the control of fetal development and pregnancy health (Aim 1). This

will be helpful in developing advice for couples who want to get pregnant or are having problems with maintaining a healthy pregnancy. By understanding the pathways altered by the environment of the parents, this work will also be helpful in identifying biological markers that can be used to screen for, diagnose and treat pregnancy complications. It will also provide information on the importance of environment/lifestyle of the mother, father, or both in determining the life-long health outcomes of the offspring (Aim 2) and the mother (Aim 3) after pregnancy. This will provide much needed information on the pathways that link the environment/lifestyle of the parents to the increased risk of the child to develop diseases like diabetes and heart disease in the years after birth. This will be useful in identifying people at risk of ill health from poor conditions during pregnancy and early life. It will also help us to know whether such conditions are already set during pregnancy, or arise after pregnancy (say when combined with poor lifestyle factors after birth). These findings will be valuable for the advice given to couples who are pregnant or planning pregnancy. Our experiments are designed in a way that we can also measure the extent to which a poor functioning placenta (an organ that develops to nourish the fetus) may play a part in the negative impacts of poor conditions in the parents on pregnancy and later health. These experiments will likely identify biological indicators that could be measured in the placenta at birth to help predict risk of diseases in later life. Identifying individuals at high risk would allow for closer monitoring and more targeted and earlier interventions to prevent diseases from developing. Finally by testing if interventions given to the mother,

father or both, protect the fetus/offspring from developing ill health (Aim 4), we will find ways to prevent the harms caused.
For example, our results will help people who work in health care to provide more specialised lifestyle advice and improve pregnancy health outcomes. We will also know if these beneficial outcomes are mediated by changes in the placenta, which will allow further development of treatments. For example, targeting therapies to the placenta to avoid any side effects on mother and fetus. All four aims will provide valuable information at many biological levels (cells, molecules, tissues and whole body) about which and how the environment/lifestyle of the mother and father instruct pregnancy outcomes and the life-long health. In the long-term, the outputs from this work are likely to reduce health care costs, the pain and suffering of affected individuals, and raise awareness of the role each parent plays in determining life-long health and disease risks, more generally.

For all the aims, the outputs of the programme of work will largely be in the form of scientific data which will be published as papers in high quality leading journals that are read by scientists and clinicians. The findings will also be presented at key conferences across different scientific fields which both scientists and clinicians attend. These activities will ensure the widest possible audience for the work. In addition, findings will be presented and discussed in lectures to undergraduate students. The findings will also be discussed with people in the media, politics, health care, companies, and at outreach events attended by members of the public.

Who or what will benefit from these outputs, and how?

The results generated in this project will have wide-reaching benefits for many scientific groups (including scientists and clinicians who study physiology, metabolism, reproduction and development) and will have great clinical, and public health relevance. Data generated will be used by researchers, including ourselves, clinicians (medical and veterinary surgeons), other health care professionals (for example, those working in assisted reproduction, midwives, nurses, health visitors) and potentially, in the long-term, by professional and government agencies (e.g. World Health Organisation, General Medical Council, Royal College of Veterinary Surgeons) and pharmaceutical organisations who may develop policies, diagnostic tests and therapies to combat sub/infertility, pregnancy complications and later life health problems based on this study. In the long-term the outputs from this work are likely to reduce health care costs, the pain and suffering of affected individuals, and raise awareness of the role of the mother's and father's environment/lifestyle for life-long health and disease risks, more generally. The data generated will be used to:

- Design experiments by ourselves and colleagues in the scientific community in a wide range of fields, to further optimise human health, to reduce the burden of disease and to reduce, refine and replace the use of animals in experimental procedures.

- Identify environment/lifestyle factors in the parents that do and do not have potential health risks for the mother and offspring.

- Initiate human population and clinical studies in humans based on findings from our experimental studies in mice.

- Develop tools to diagnose, treat and prevent pregnancy complications and their related long-term effects.

- Revise life-style advice given to parents planning/currently pregnant.
- Provide advice to clinicians and other health care professionals on how to monitor, treat or intervene.

How will you look to maximise the outputs of this work?

In the proposed work, we will ensure that we can obtain multiple datasets from each animal. In particular, we will make as many measurements as possible on an individual animal. At the end of the experiment, we will also obtain plenty of tissue and blood samples that can be used for additional projects. For e.g., tissue and blood samples may be used to develop a new method, answer a question outside of the direct scope of this project, or used to generate data for new work. We will maximise the outputs of this work by making data and methods freely available to others and we will also ensure rapid communication of findings through 1) open-access publication in the leading journals, 2) presenting our findings at key local, national and international conferences across different fields relevant to the research and at which scientists, clinicians and policy makers are in attendance, 3) taking opportunities to present and discuss findings in lectures to undergraduate students, with the media, politicians, clinicians, industry representatives and at public outreach events and 4) further outreach activities including publications in magazines, news articles, blogs, and via twitter / social media feeds.

Species and numbers of animals expected to be used

- Mice: Mice: 23,250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project requires the use of animals in an integrative study of the body during development, pregnancy and later life. It requires exposing the animal to a genetic alteration and/or a change in its environment and measurements made on live animals and tissues and blood taken subsequently. It requires the study of the parents, fetus and offspring. Hence, this work cannot be fully replicated in vitro or in a cell culture system. This work would provide vital information on the importance of the environment of the mother, father and both for fetal development, pregnancy wellbeing, and offspring health. They are also important for knowing if interventions/treatments can improve pregnancy outcome and life-long health.

Mice have been chosen as the best non-human model for this study for several key reasons. This includes the knowledge that the patterns of egg/sperm and fetal development in mice are similar to humans. The genetic make-up of common laboratory mice allow us to undertake very precise investigations into the complex interactions between genes and the environment in determining pregnancy and offspring health. Such detailed study of these mechanisms in humans would be considered unethical. The structure of the placenta (the organ that develops to nourish the fetus) in mice is also similar to humans. Furthermore, the mouse also has a short gestation and short lifespan compared to other species which means that the proposed work can be completed within a shorter timeframe so that improvements in human health can be realised more rapidly.

The mouse is also large enough to allow analyses of body functions (physiology), yet is small enough to permit complete assessment of tissue structure, which is not possible in larger animals. Finally, the expression of specific genes and the environment of the parents modified in a controlled way. This allows precise, highly refined information into the processes regulating fetal development, placental function, pregnancy health, and offspring wellbeing

Typically, what will be done to an animal used in your project?

Mice will be exposed to a genetic manipulation and/or a change in their environment. Manipulation of the environment for males will occur prior to mating and for females will occur prior to mating, during pregnancy and/or during lactation. Different combinations of matings will be set-up to assess the separate and combined impact of manipulations of the mother and father on pregnancy health, fetal growth and placenta function. Additional combinations of matings will be set-up to assess the health of the offspring and mother after pregnancy will be studied. Other work will involve testing the effectiveness of interventions/therapies in mice.

Animals in this project may be subjected to

•Genetic manipulations that alter key pathways within the whole animal or within certain cells/tissues (e.g. placenta), and tissue sampling (normally a ~2mm skin sample taken from the outer edge of the ear) to determine genetic status. This will involve breeding and maintenance of genetically altered mice and will tell us which genes/processes are important for healthy pregnancy outcome, as well as predisposition and protection of the mother and offspring from disease. The genetic manipulations will have been chosen to study development and physiology and animals will be exposed to mild severity (causing no more than short-term, mild pain, suffering or distress to the animal, and with no significant impairment of their overall wellbeing or condition). We do not plan to generate new genetically altered lines.

•Environmental manipulations to the mother and/or father prior to mating, during pregnancy and/or during lactation. These environmental manipulations include dietary interventions (such as under- nutrition or diets containing high sugar and/or fat) and reducing oxygen levels (inhalation hypoxia). Animals may be exposed to the dietary manipulation for up to 12 months or low oxygen (inhalation hypoxia) for up to 22 days, but typically for a much shorter duration. This will inform which specific environmental conditions in each parent impact pregnancy outcomes and life-long health of the mother and offspring. The environmental manipulations have been chosen to study physiological responses, rather than extreme exposures/stressors and are not expected to exhibit any harmful phenotype.
However, some animals will be exposed to moderate (instead of mild) severity, as they will be exposed to environmental manipulations and experimental procedures when they are pregnant.

•Environmental manipulations in the offspring and/or mother after birth. These environmental manipulations include dietary interventions (such as under-nutrition or diets containing high sugar and/or fat) and reducing oxygen levels via inhalation hypoxia. Animals may be exposed to the dietary manipulation for up to 12 months or hypoxia for up to 22 days, but typically for a much shorter duration. The environmental manipulations have been chosen to study physiological responses, rather than extreme

exposures/stressors and are not expected to exhibit any harmful phenotype. These animals will largely be exposed to mild severity.

•Drugs including anaesthetics, hormones, stimulators and inhibitors of hormones and growth factor pathways, as well as imaging substances given to study how the body functions at specific times. Administration will either be through injection into vein, body cavity or under skin, in their diet/drinking water, or by the surgical implantation of a small infusion device (osmotic minipump) under the skin.
Other than in terminally anaesthetized animals (where they will not be recovering from deep sleep prior to humane killing), the volume, route and number of administrations of the substance used will be the minimum needed to meet the scientific objective. These animals will be exposed to mild severity.

• Drugs including antioxidants (substances that can prevent or slow damage to cells caused by unstable molecules that the body produces), nutrients, and other possible agents with therapeutic potential given to study their effectiveness in improving pregnancy outcomes and preventing disease. Administration will be either through injection into vein, body cavity or under skin, in their diet/drinking water, or via surgical implantation of a small infusion device (osmotic minipump) under the skin. Other than in terminally anaesthetized animals, the volume, route and number of administrations of the agents used will be the minimum needed to meet the scientific objective. These animals will be exposed to mild severity.

•Embryo transfer experiments to begin to discriminate between the effects of changes in the eggs/sperm of the parents from those caused by a poor environment in the mother after mating. This will involve examining the development of the embryo in culture prior to implantation and then transferring embryos to recipient females with analysis of pregnancy and offspring health outcomes. We may also study the mothers after pregnancy, to see what impact there may be on her disease risk. As far as possible, the embryo transfer experiments to recipient females will be performed by non- surgical methods to minimise impacts on the animal.

•Alone housing for a maximum of 4 weeks to allow precise analyses of body functions (e.g. metabolism and food intake) or changes in food intake (including for pair-feeding when needed as an additional control group).

•Blood sample collection from an easy to access vessel.

•Fasting, so that precise measures of body functions can be determined (no more than 6 hours in pregnant animal and no more than 16 hours in adult mice).

•Litter standardisation or cross-fostering of offspring to different mothers. In the case of cross-fostering, this will occur in the first 48 hours after birth to maximise the success of fostering. We will monitor the mother until nursing behaviour has been displayed, or for a maximum of 1 hour. Cross fostering will take place in the home cage. The foster mother and pups will be handled with clean gloves to reduce the introduction of human odours and the pups will be mixed with the nesting material from the foster mother's cage.

•Non-invasive scanning/imaging or monitoring assessments to determine body composition, behaviour, metabolism and cardiovascular function on one or more occasions (e.g. time-domain nuclear magnetic resonance [TD-NMR], metabolic cages for

up 72h, behavioural testing, ultrasound, tail cuffs; up to 10 over a 12 month period, but no more than one occurrence in pregnancy aside for TD-NMR).

•At the end of the experiment, each animal will be killed using the most humane method that is possible that does not prevent us obtaining good scientific data.

What are the expected impacts and/or adverse effects for the animals during your project?

- Genetically altered mice used in this programme of work are not expected to exhibit any harmful phenotype (e.g. causing more than short-term mild pain, suffering or distress to the animal, and/or with significant impairment of their overall wellbeing or condition) but may show changes in their pattern of growth and development.

- The environmental manipulations have been chosen to study physiological responses, rather than extreme exposures/stressors and are not expected to exhibit any harmful phenotype. The environmental manipulations may cause reduced weight gain (e.g. with under-nutrition or with hypoxia which can be linked to reduced food intake), increased weight gain and/or mild features of metabolic disease (obesity, insulin resistance). These effects are no longer apparent when the manipulation is removed and/or often alleviate after a few days of the exposure (e.g. reductions in food intake normally only occurs during the first couple of days in hypoxia exposed animals). However, the effect of environmental challenges may be increased or seen for a longer period after cessation of challenge in mice with genetic alterations.

- There may cumulative impacts for animals undergoing different experimental procedures during pregnancy (e.g. reduced weight gain in pregnancy).

- If mice with gene alterations, environmental exposures and/or different experimental procedures during pregnancy exhibit any harmful phenotype, they will be humanely killed, or in case of individual animals of particular scientific interest, advice will be sought promptly from the local Home Office Inspector.

- Mice showing evidence of suffering that is greater than minor and transient or in any way compromises its normal behaviour will be humanely killed.

- Mice displaying weight loss that is not an expected outcome from the manipulation (altering growth trajectories that reaches 15% against age matched controls) will be humanely killed.

- Any mouse displaying rapid weight loss, measured on a daily basis, up to a maximum of 15% within a 7 day period, will be humanely killed.

- Any mouse displaying intermittent hunching or abnormal stance or reduced activity for a period of time not to exceed 24 hours, will be humanely killed.

- Any mouse displaying altered respiration (intermittent abnormal breathing patterns exceeding 1 hour) will be humanely killed.

- Any mouse displaying suppressed activity (subdued or isolated behaviour and reduced response to provocation for a period not to exceed 5 hours), will be humanely killed.

- Although highly unlikely, some animals may have an altered immune system making them more susceptible to infection. Animals with an altered immune status will be housed in a barrier environment thereby minimising the likelihood of compromising health.

- Mice are not expected to experience any lasting harm from being given hormones, stimulators/inhibitors of hormones/growth factor pathways, other drugs used in these studies or imaging substances. Novel drugs and other possible therapy agents will be tested to make sure they do not cause unwanted side effects that are harmful to the mice.

- Single-housing can be stressful for mice, but we will minimise the time each animal is housed alone. Where possible, animals will be re-housed in groups with their original cage-mates following a period of single-housing.

- In the unlikely event that pups are rejected in cross-fostering experiments the dam and her litter will be humanely killed.

- Animals are expected to make a rapid and unremarkable recovery from a surgical procedure involving anaesthesia within two hours. In the uncommon event that they fail to do so, or that the animal exhibits signs of pain, distress or significant ill health, they will be humanely killed unless, in the opinion of the Named Veterinary Surgeon, such complications can be remedied promptly and successfully using no more than minor interventions or a programme of enhanced monitoring and care is instituted until the animal recovers fully. Any animal not fully recovered from the surgical procedure within 2 hours (eating, drinking and returning to their normal behaviour) will be humanely killed. In the case of wound dehiscence, uninfected wounds may be re-closed on one occasion within 48 hours of the initial surgery.

- Animals anaesthetised for imaging (e.g., ultrasound) may feel disorientated as the anaesthetic wears off, but will experience no pain or lasting harm. TD-NMR scanning for body composition analysis and application of tail cuffs for blood pressure monitoring requires restraint, which can be stressful to mice, but animals will be trained / acclimatised to the equipment beforehand to reduce the stress.

- Previous work has largely assessed the effect of single parent manipulations on offspring growth, cardiovascular and metabolic physiology. We therefore do not know if there may be a stronger effect when environmental exposures are given to both parents (e.g. more severe body fat/weight gain and insulin resistance). Thus, careful monitoring of all offspring will be undertaken to ensure that they do exhibit any harmful phenotype. If they do exhibit an unexpected harmful phenotype, offspring they will be humanely killed, or in case of individual animals of particular scientific interest, advice will be sought promptly from the local Home Office Inspector.

- There can be impacts on the animals when they are undergoing different experimental steps when pregnant. However the number of steps will be kept to a minimum, and mice will be closely monitored to ensure there is no evidence of suffering that is greater than minor and transient. If so, they will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mice
- Mild 60%
- Moderate 40%
- Severe 0%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project requires the use of animals in an integrative study in vivo. It requires measurements to be made in vivo with subsequent in vitro analyses of tissues of mice and their offspring following environmental/genetic manipulation. It also requires measurements to be made in vivo with subsequent in vitro analyses in mice and offspring to test the efficacy of interventions/treatments in improving pregnancy outcome and life-long health. The outcomes of these studies are multi-factorial involving interactions in vivo between genes of the parents, the fetus, placenta and mother during pregnancy and between the mother and neonate/offspring after birth. Its study therefore requires the use of living animals rather than isolated cells, tissues and organs. However, ex vivo and in vitro analyses (e.g. tissue culture, isolated organ function) subsequent to the in vivo measurements will allow us increase the data obtained and provide the comprehensive, integrated approach (from the gene to the systems level) that we are seeking in our programme of work. Consequently, answering the questions that we are addressing cannot be achieved in any other way than by using animals in vivo.

Which non-animal alternatives did you consider for use in this project?

The sole use of cell culture and single organ systems would not permit investigation of the consequences of parental manipulation and the role of the placenta in maternal wellbeing, fetal growth and in the programming of disease in later life envisaged in the proposal. However, as a complementary approach, we will endeavour to use placental explants, trophoblast stem cells or cell lines, and the recently developed placental organoids as a way to reduce animal usage, in particular to help define precise molecular mechanisms and provide causal relationships (through, for example, genome editing in these culture systems). We can also use primary and established cell lines to test the contribution of specific molecular changes/pathways identified to be involved in the environmental programming of phenotype, but this can only happen after they have been identified from employing experimental manipulations in vivo. Current links with clinical researchers and elsewhere will help to translate findings generated under this licence, to humans.

Why were they not suitable?

Even with the best efforts in adapting experiments on cells, organoid and isolated organ systems in vitro, these set-ups offer limited benefit for understanding mammalian

physiology and developmental processes in the later stages of pregnancy. In vitro set-ups do fully recapitulate the complex in vivo environment which changes over time with internal and external cues and involves dynamic communication between different organ systems, and signalling by circulating hormones, metabolites, and growth factors.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers were informed on our previous work (published and unpublished/pilot experiments obtained over several years). This takes into account pregnancy success rates, numbers of small litters and natural neonatal survival rates.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We carefully consider each animal and what information it can provide, to maximise the possible tissue and data collection and reduce animal use. For example, for pregnancy studies, we retrieve the placenta, blood, and multiple organs from the mother and fetus, as much as possible, so that they can be used for multiple analyses across different projects. This reduces the numbers of animals used overall, increases the amount of data obtained from a single pregnant animal and allows us to examine links between the mother, placenta and fetus, thereby enhancing the quality of the information produced.

We also very carefully consider which control groups are necessary to address the scientific question and use power calculations as much as possible to ensure we will use an appropriate number of animals.

Assistance with experimental design was also obtained via web-based sources through the National Centre for the Replacement, Refinement and Reduction in Animals in Research (www.nc3rs.org.uk/experimental-design) and Prepare (https://norecopa.no/PREPARE).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Strains (types) of laboratory mice with known genetic status will be bought from known breeders or bred in-house, which reduces variation between animals. As far as possible, we ensure the breeding of colonies optimises the animals generated whilst minimising animal wastage. Only the number of breeders needed to generate the required number of offspring for a particular study will be set-up and scaled up or down, as required, as the litters are born and used. We will use control mice that are at the same age as the experimental mice and will use littermates (siblings) as much as possible. We will use mice that are genetically identical (i.e. sharing the same genetic background).

We will ensure the conditions of mouse husbandry are highly controlled (temperature, humidity, diet) and only parameters to be tested (e.g. diet) will be altered in a controlled way. Measurements will be taken at the same time of the day (e.g. to account for circadian effects on body functions). Where possible, initial checks of animal fertility and ability of the mother to nurse her pups will be made before the experimental pregnancy and particularly, when there is follow-up of offspring and maternal health.
We will randomly allocate animals to each treatment group and outcome measure. In designing experiments, controls and experimental groups will be run at the same time to avoid seasonal differences. We will perform pilot experiments, for e.g. to check the effectiveness of new therapies. Typically, controls and experimental groups are first compared with 4 animals in each group. If statistical significance is unlikely, no further animals will be used.

To maximise data gained from each animal, we will collect a large number of measures, as well as tissues and ample blood to provide a data and tissue/blood bank. This will enable us to carry out additional studies, including by collaborations - without having to use new cohorts of animals.

We also make extensive publication searches to ensure that we have a comprehensive, systematic knowledge of the literature in designing new studies and in identifying potential new collaborations that can use our archived material. We also go back to previously collected data from our animals, as it helps us to design new experiments, and may answer the novel questions with no extra mice needed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice have been chosen as the optimal non-human model, for this study for several key reasons including i) their patterns of egg/sperm development, and embryonic and fetal development are similar to humans, ii) the genetic make-up of common laboratory mice permits unparalleled investigation into the complex environmental effects on offspring health and the pathways involved, iii) mouse models have become firmly established for analysis of environmental mechanisms involved in 'Developmental Origins of Health and Disease' applications and, iv) they have relatively short gestations and lifespans, which allows critical stages of development to be targeted and the effects of aging assessed, and v) the wide availability of modern gene deletion and knock-in technologies means mice are ideal for discovering the role of specific genes in mediating the effects of environmental influences.

The following methods will be used:

All surgery with recovery will be carried out under sterile conditions (free from harmful microorganisms). All animals will be monitored on a regular basis to ensure their welfare

and protocols usually begin with the least invasive procedures and move onto more invasive experiments when positive results are obtained. Pro-forma recording sheets will be used during surgery to monitor variables (e.g. temperature, heart rate, anaesthetic dose) and post-operatively for the current severity score, food consumption and treatments with addition of free hand comments as required (e.g. recovery times, general demeanour) to monitor overall welfare over the course of an experiment.
Routes, dosage volumes, frequencies, durations will be undertaken such that animals fully recover between administrations and will not suffer more than transient pain and distress and no lasting harm and there will be no cumulative effect of repeated administrations. In protocols involving food withdrawal in rodents, where possible food withdrawal will occur during the daylight hours, as this is better for the welfare of the animals. By archiving tissue for new studies and using equipment (e.g. metabolic cages [cages that all measurement of the animal's behaviour, food intake and body energy use], TD-NMR) with detection or recording devices that signal information and provide data without restraint, animal welfare can be maintained and data collection can be maximised while minimising the number of animals used experimentally. Of note, use of TD-NMR which is not invasive and does not require anaesthesia allows for longitudinal body composition to be measured in the same animal which reduces the numbers of animals needed. The use of metabolic cages which allows for highly resolved longitudinal and integrated assessment of metabolism, body weight and behaviour to be measured in the same animal which reduces the numbers of animals needed. The use of ultrasound which requires anaesthesia enables one to obtain longitudinal information on maternal-fetal blood characteristics and fetal development in the one animal which reduces the numbers of animals required.

Why can’t you use animals that are less sentient?

Species that are less sentient or at an immature life stage would not allow us to address our key scientific question related to the role of the placenta in determining parental influences on pregnancy and life-long health. Less sentient species like flies or worms are not viviparous and therefore do not have a placenta or gestate. We use terminally anaesthetised mice for some procedures, and studies on terminally anaesthetised animals can help to identify physiological manipulations and/or intervention treatment in the preliminary phase. However, terminally anaesthetised animals do not provide the time scale or ability to track the developmental changes that underlie the longer-term programming of phenotype.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

- Continually read papers in the field, including those which describe different methodological approaches to assess whether end-points measured should be adapted.

- Close supervision and accurate training records of personal licence holders to ensure their use of experimental animals always complies with the Home Office regulations, the project licence and to ensure there is minimal welfare costs for the animals.

- Close engagement with the Named Persons who might be able to help to ensure there is minimal welfare costs for the animals.

- Ensure and regularly evaluate records of animals used in experimental procedures, including post-operative care records and health monitoring of animals exposed to an environmental challenge to see whether any procedures need to be refined.

- Carefully record and examine mice in experimental protocols to ensure they do not reach the end of the severity band to which they are covered, and if so act immediately to ensure these are accurately reported and to avoid this in the future.

- Explore any unexpected deaths of any mice in experimental protocols to see what may have happened so that it may be avoided.

- Employ metabolic cages, TD-NMR and other scanners of genetically modified and environmentally challenged mice to refine animal use. Metabolic cages allow us to non-invasively and precisely evaluate many aspects of an animal's body functions, including food and water intake, energy use, behaviour, activity levels, and body composition and weight while they are normally moving around in the cage. Normally these assessments would be made on separate animals.

- As far as possible, we will use cage enrichment items (tubes) and group house animals, so that the quality of life of the animals is maximised. We will supply soft bedding and mushy food as necessary and in addition to weighing animals, we will monitor body condition scores (Ullman-Cullere and Foltz 1999 Lab Animal Sci) to provide further information on the wellbeing of the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

- Assistance when designing experiments by using web-based sources through the National Centre for the Replacement, Refinement and Reduction in Animals in Research (www.nc3rs.org.uk/experimental-design), ARRIVE (Animal Research: Reporting of In Vivo Experiment, guidelines for preparing publications; https://www.nc3rs.org.uk/arrive-guidelines) and PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence; https://norecopa.no/PREPARE and https://www.ncbi.nlm.nih.gov/pubmed/28771074)

- Laboratory Animal Science Association (LASA) guiding principles documents for aseptic technique for any surgical procedures (https://www.lasa.co.uk/current_publications/)

- Continually read papers in the field, including those which describe different methodological approaches and the latest advances to assess whether study design or end-points measured should be adapted.

- Close engagement with Named Persons and the Named Information Officer at my Establishment.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

- Relaying information, ideas and strategies to refine our study approaches by discussing with members of the laboratory

- Regularly checking in with researchers in the laboratory to see if there are any queries relating to the licence and study designs.

- Regularly checking my Establishment website and search tool to check for any advances in the 3Rs and implementing these.

- Make a regular practice of reviewing our procedures to improve them from both welfare and scientific perspectives

- Continually read papers in the field, including those which describe different methodological approaches and the latest advances to assess whether study design should be adapted.

- Making use of the NC3Rs website pages https://nc3rs.org.uk/resource-hubs, 3Rs tools in-house, and externally resources, such as Norecopa https://norecopa.no/databases-guidelines

200. Self-Reporting Arterio-Venous Grafts in Pigs

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Chronic Kidney Disease, Arterio-venous Implant, Management

Animal types	Life stages
Pigs	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To test the safety and efficacy in pigs of an implanted device that allows the openness (or "patency") of an artificial blood vessel (a fistula, or a graft) connecting an artery and a vein. Arterio-venous fistulae (AVF) or grafts (AVG) are surgically created to allow the removal and return of large blood volumes during dialysis sessions in human patients with chronic kidney diseases (CKD).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Renal dialysis (which is required 3 - 4 times per week in people with CKD) relies on repeated access to veins, or more commonly, AVFs, or AVGs. However, all access

attempts cause some degree of AVF damage resulting in progressive scarring and a reduction in the AVF's internal diameter (stenosis).
Eventually the resultant blood flow becomes so inadequate that dialysis is no longer possible. This not only delays urgent treatment but requires another AVF (or AVG) to be created.

What outputs do you think you will see at the end of this project?

The study will reveal the safety and efficacy (or otherwise) of a device used to non-invasively, i.e., painlessly, indicate the patency of an AVF or AVG. The study will at least provide information allowing device improvement. At best, it will indicate suitability for the first human trials. The study has the potenial to generate new information, publications and an effective safe device.

Who or what will benefit from these outputs, and how?

Successful safety and efficacy testing in pigs will eventually allow the device to be used in humans for the first time. This has the potential to improve the management of CKD in the large number of people suffering with this condition world-wide.

How will you look to maximise the outputs of this work?

The outputs of the work, if successful, will be published, facilitating in turn collaborative multi-centre "first in human trials".

Species and numbers of animals expected to be used

- Pigs: 15

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The size and (to a lesser extent) the relative location of arteries and veins for the creation of AVF are similar in adult pigs and humans. The reactions to blood vessel damage and blood clotting are also similar. Methods for creating arterio-venous fistulae in pigs have been described. Surgery will be conducted on dead pigs before the first live pig study is attempted.

Typically, what will be done to an animal used in your project?

In the first, non-recovery phase, pigs will be loaded individually onto an approved animal trailer and transported by road (approximately 10 - 30 minutes) to the facility. Pre-anaesthetic medication (to which hyaluronidase may have been added) will then be given by intramuscular (IM) injection. This will typically produce sufficient central nervous depression, anxiolysis and muscle relaxation for the animal to be lifted onto, and moved by trolley - without resistance - to the anaesthesia induction area. Here the level of sedation will be deepened (using inhaled anaesthetic) to produce conditions whereby there is no observable response to attempted venipuncture, e.g., ear-flapping +/- head shaking. When

the cannula is in place anaesthesia will be induced, usually by a combined IV and, or inhalation technique. Thereafter, continuous and close monitoring of responses to (normally) painful stimulation will ensure the attending anaesthetist is always aware of the animal's anaesthetic depth, whilst the immediate availability of additional doses of rapidly-acting anaesthetics pre-located in a venous cannula will ensure the rapid restoration of oblivion if required. The study will end once all objectives have been achieved as far as is possible within the permitted time limit. The animal will then be terminated whilst anaesthetized at the end of the study and not released for post mortem examination, or disposal, until physical examination confirms the animal is dead.

In the second, recovery phase, pigs will be loaded (typically in pairs) onto an approved animal trailer and transported by road (approximately 10 - 30 minutes) to the facility.. They will then be acclimatized for one week pre-procedure during which they will be trained (using feed) to enter the proprietary weighing crate where post-procedurally, the wireless output of the implanted transmitter will be captured and recorded. They will also be injected intramuscularly (IM) with anti-coagulant drugs.
They may also wear a dummy cannula taped to one ear to acclimatize pen mates to an object of interest (and interference). Pigs may be housed singularly, but in visual, auditory, olfactory but limited tactile contact. Thereafter, initial experiences will be as above except pigs will be allowed to recover from anaesthesia. Before this happens one or more type of analgesic (pain-killing) drug will be given. An attendant will remain with each animal until it is fully recovered from anaesthesia and is eating & drinking normally. After this, regular examinations will occur to assess general indications of pain and, or discomfort. The wound will also be examined at these times. Signs of pain or other complications will be treated using drugs injected into the muscle. Antibiotics will also be injected IM. Blood may be sampled periodically during this time through the indwelling cannula to evaluate the blood's ability to clot. Pigs in phase 2 will be euthanized using an overdose of anaesthetic a maximum of 28 days after the implant surgery. They may be euthanized before this time if untreatable complications arise that compromise their health and welfare or study objectives.

What are the expected impacts and/or adverse effects for the animals during your project?

Pigs in the first (terminal) study will not recover from anaesthesia so will not experience anything worse than transport from the home farm and, or a single intramuscular injection.

Pigs in the recovery group may experience modest post-operative pain but this will be controlled with analgesic drugs. Particular attention will be paid to difficulty in eating and swallowing associated with surgery, and steps taken to minimize this, e.g., altering trough height. The implant will be tested at least once daily in the weighing crate so offering an ideal opportunity to measure changes in body mass. Regular frequent examination will also detect signs of abnormal bleeding (bruises).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

A maximum of 5 pigs will undergo non-recovery studies whilst a maximum of 10 will undergo studies of "moderate" severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The need to study the implant's functionality and interaction with the numerous pathophysiological processes of interest, e.g., coagulative, immune, inflammatory, and vasomotor, for up to 28 days under conditions of pulsatile blood flow precludes in vitro testing.

Which non-animal alternatives did you consider for use in this project?

The device has been bench tested using explanted primary vascular smooth muscle cells, endothelial cells and blood clots (and shows a consistent spectral signature predictive of increased cellularity over time) but no non-animal model exists or would provide the extent of information required.

Why were they not suitable?

The explanted tissue constructs cannot yield the type of information necessary for the first use of the device in humans.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The surgical (implantation) technique will be perfected in preliminary cadaveric studies using pigs of similar size.

Non-recovery studies will be conducted on two animals to test the implant's in vivo performance.

The device will then be implanted in four pigs under general anaesthesia. These will be allowed to recover from anaesthesia after which the implants performance will be evaluated for up to 28 days.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Explanted tissue was first used for bench testing and calibrating the implant.

Pig cadavers will be used to optimize implantation surgery in order to minimize the number of pigs required in the terminal and recovery phases of the study.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

If possible, the cadaveric studies will be conducted on suitably sized animals killed after the completion of other studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

A size-relevant (large) animal model, i.e., commercial juvenile / adult pigs, is required to test the safety and efficacy of this device before it is tested in adult humans.

The study is staged, i.e. will progress from cadaveric, through non-recovery to recovery studies to ensure both surgical techniques and device performance are optimized before application in animals scheduled to recover from the anaesthetic.

Why can’t you use animals that are less sentient?

Less sentient species would not be size-relevant.

Terminal studies lasting 28 days (which are required for meaningful implant evaluation) would be infeasible.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The study is staged, i.e. will progress from cadaveric, through non-recovery to recovery studies to ensure both surgical techniques and device performance are optimized before application in animals scheduled to recover from the anaesthetic.

The device has been designed to optimize chances of successful animal testing, being powered by one "active" and one "back-up" battery. In the unlikely event the former fails, the latter will be recruited to ensure the experiment is completed.

Animals will be acclimatized in pairs in an enriched environment in which post-implantation testing will occur. Pairing will continue post-implantation providing study outcomes are not compromised by implant interference.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The PREPARE Guidelines and checklist will be reviewed and completed by the applicant and all other persons involved in the proposed study.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am an active member of a number of ethics committees/interest groups and laboratory animal associations including 1) The local AWERB; 2) EthicsFirst, an online forum promoting animal ethics; 3) The Animal Welfare, Science, Ethics and the Law Veterinary Association; 4) The Laboratory Animal Veterinary Association; 5) The Laboratory Animal Science Association.

I subscribe to the online newsletters of: 1) the NC3Rs; 2) The Nuffield Council of Bioethics; 3) Understanding Animal Research.

201. Skeletal Tissue Engineering

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Skeletal, Tissue engineering, Biomaterials, Stem cells, Repair

Animal types	Life stages
Mice	adult
Rats	adult
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aims of the project are to use tissue engineering to develop the best conditions for cell and tissue growth on biomaterials to repair a broken or diseased skeleton. Our rationale is centred on the urgent need to develop materials capable of activating and growing both blood vessels and bone to help patients repair their damaged and diseased skeletal tissue.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

With an increasing ageing population, skeletal tissue loss due to injury or disease is rapidly growing. This significantly impacts the quality of life for the patient over time and has socio-economic costs for healthcare providers. For example, each year in the UK there are over 50,000 primary hip replacement operations at a cost in excess of £350million. Hence, for reconstructive bone surgery and fracture repair, the need to develop better techniques and alternative bone therapies is vitally important.

From a patient's perspective, the ultimate goal is to repair and replace their damaged skeleton with bone material, harvested with minimal complications from their own skeleton. However, the current clinical approach for the treatment of bone defects and non-unions is bone grafting or metal implants, which both have significant drawbacks. The combination of a bone stimulating material combined with bone tissue generating cells to produce a therapy would be an invaluable surgical option, reducing both complication rates occurring in patients undergoing bone replacement surgery. Therefore, generating materials that can help bones repair themselves will be invaluable for patients and animals suffering from diseases and fractures affecting the skeleton. These include non-healing bone fractures and weakened bones due to diseases like osteoporosis and bone cancers.

What outputs do you think you will see at the end of this project?

The proposed models being used in these studies will help us to gain new insights in the development of tissue regenerating biomaterials to rebuild broken bones

Specifically.

We want to use cutting-edge technology to figure out how biomaterials stimulate bone cells and tissue to grow and repair, and then use these imaging and stimulating devices to direct biomaterial formulations to the exact location of the bone fracture.

We want to create new biomaterial constructs that can stimulate bone regeneration so that we can provide new and better treatments for people who have fractured bones or bone lost due to other diseases such as cancer.

We will present our findings at scientific and medical conferences and we will publish these outcomes in peer-reviewed scientific journals.

Finally, any new procedures/methodologies improved welfare settings developed throughout the period of the project license we will aim to publish and share among the scientific community to benefit both human and animal patients.

Who or what will benefit from these outputs, and how?

As with all our research goals the aim is to develop improved therapeutic strategies and products for patients who suffer from skeletal diseases and bone trauma injuries. We envisage that these project protocols and novel biomaterials have the potential to provide new treatments to help rebuild bones and skeletal tissues. In addition, the findings from musculoskeletal research can be applied to the veterinary field due to similarities in orthopaedic conditions between people and animals, leading to novel applications in a ‘one health’ approach.

Beneficiaries will include:

- Patients and animals suffering from broken bones and skeletal diseases.
- Healthcare providers.
- UK, EU and worldwide tissue engineering/biotechnology companies involved in tissue regeneration, stem cell biology or developing innovative tissue scaffold technologies,

- the academic community in the generation of new protocols and avenues for skeletal tissue regenerative research.

Many materials we use in our studies are biocompatible and currently used in clinical practice for other applications. Modifying them to enhance their properties can result in new therapies in a relatively short time frame, benefitting patients due to the known track record of efficacy and safety. However, with the complex materials proposed the final outcomes for patients may require months to years of investigation due to thorough testing to ensure safety and efficacy prior to use in the clinic.

When these new treatments become available, we envisage growing the tissue constructs in the laboratory and transferring the regenerated samples to the patient in theatre. Ultimately, we believe this work will be translated to the clinic and benefit patients within the National Health Service and the wider medical community in the area of musculoskeletal repair. Alternatively, the development of materials that exploit the regenerative potential of the patient's own repairing cells would minimise costs and hasten the material's therapeutic implementation.

In addition, the information from this project, including the study protocols and techniques will be made freely available via publication in peer-reviewed journals, in order to benefit patients, other researchers, doctors, vets, and pharmaceutical companies who are involved in the development and assessment of novel therapeutics which target the repair and regeneration of skeletal tissues due to disease and injury.

How will you look to maximise the outputs of this work?

Training & Collaborations

The team undertaking the research will acquire skills and expertise in bone repair, blood vessel and skeletal stem cell biology, biomaterial development, imaging techniques in real-time such as 3D x-ray scanning and 3D optical imaging. Training will be achieved through an extensive local network of multidisciplinary collaborations within the University, including: Clinical Orthopaedics, Biomedical Research Facility, Biomedical Imaging Unit and the Histochemistry Research Unit, and established national and international collaborations in regenerative medicine.

In addition, the protocols and research work will be communicated to our Student & Postdoctoral Interactive Network to provide new students and postdoctoral researchers information regarding the models available for research projects in our University and to foster collaborative projects. We are part of the national and international Regenerative Medicine collaborative group networks where we share our methods and results from acellular materials that will be available to be tested in skeletal and other tissue/organ regenerative models.

Education & Public engagement

The information and findings generated from this project will be presented through the scientific community by presentations at national and international scientific research meetings. We will also communicate findings to the public through outreach activities such as the University Science Day, and laboratory open days for GCSE and A-level students in tissue regenerative medicine.

Progress of the study and results will be regularly presented at ongoing teaching events and public lectures. The press office of the University will also publicise the results.

Species and numbers of animals expected to be used

- Mice: 660
- Rats: 120
- Rabbits: 60

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Whilst all attempts are made to reduce the use of animals by using in vitro methodology (in vitro experiments on human and mouse skeletal stem cells, explant studies and organotypic models), it is inevitable in work of this nature that in vivo investigations are required to be undertaken. The animal models detailed are critical to facilitate in vivo proof of concept and efficacy in an relevant biological system, not possible in humans. Adult mice and rats will be used in these investigations because they are the most reproducible and well characterised animals for (1) skeletal tissue regenerative models and (2) bone fracture models.

Bone disease and fractures may occur across the lifespan, ranging from young to old, however, it is predominantly in the adult and aged populations that the repair mechanisms in bone start to fail.
Therefore, it is expected that the vast majority of experiments will be performed in adult animals.

One of the biggest hurdles to overcome in clinical orthopaedic repair is the regeneration of large segments of bone that have been lost due to disease or trauma. We will therefore use rabbits at the final stages of these studies as this provides a larger bone defect model to test the potential of our repair materials to regenerate large amounts of bone tissue.

Typically, what will be done to an animal used in your project?

Scenario 1 (50%)

Various biomaterials, cells or substances will be either injected and assessed for localisation to the bone or will be injected/implanted under the skin of mice to assess that the agent is retained at the injection/implant site and is able to stimulate new bone formation followed by recovery. As it takes some time to make new bone the experiments will last between 6-12 weeks. Because we are seeking to localise biomaterials, cells, substances to bone locations and in particular where there is a bone deficiency, the injection studies may be shorter (1-7 days). We will assess the rate of bone formation by 3D X-ray scanning at various timepoints over the course of the study under anaesthesia. Optical imaging will also help us to pinpoint the exact location of the injected biomaterial in the bone.

Scenario 2 (30%)

Mice and rats under anaesthesia will have a portion of bone (limb-long bone) removed and biomaterials, or cells are implanted in its place during surgery or delivered by injection locally or systemically, followed by recovery. The overall limb stability will be maintained with fixation pins. This experiment will be run for 8-12 weeks as the biomaterial implant has to build the bone and then it needs to be shaped by the rodent cells to fit exactly into the broken bone site. We will assess how well this repair is going by 3D X-ray scanning, optical imaging, under anaesthesia at vary timepoints over the course of the study.

Scenario 3 (15%)

Mice and rats will have a section of bone (skull) removed and biomaterials and/or cells implanted in its place during surgery, or injected locally or systemically followed by recovery. This model is used as the skull bone develops and repairs differently from long bone fractures. This experiment will be run for 8-12 weeks as the biomaterial implant has to build the bone and then it needs to be shaped by the rodent cells to fit exactly into the broken bone. We will assess how well this repair is going by 3D X-ray scanning at varying timepoints over the course of the study.

Scenario 4 (5%)

Rabbits through a surgical procedure will have section of bone (forearm) removed and biomaterials implanted in its place followed by recovery. This experiment will be run for 8-12 weeks as the biomaterial implant has to build the bone and then it needs to be shaped by the rabbit cells to fit exactly into the broken bone. We will assess how well this repair is going by 3D

X-ray scanning/optical imaging, under anaesthesia at vary timepoints over the course of the study.

In addition to the above scenarios

1. the animals may receive an injection of bone inducing factors to localise to the bone defects to enhance the repair.

2. The animals may be subjected to a scanning device, such as ultrasound, in order to improve the implanted biomaterials' ability to promote bone healing under anaesthesia. We will use ultrasound because it is routinely used in the clinic and can activate certain biomaterials by pressure waves to release bone repairing factors (locally at a bone fracture site) without any surgical interventions for the patient.

What are the expected impacts and/or adverse effects for the animals during your project?

In 2 of the protocols the animals will exhibit transient mild pain. In the other 3 protocols (the bone fracture models) animals will experience moderate short term pain due to the procedure. With the limb defects there will be a 12-24 hour period where the mice will limit load bearing on that limb. Normal movement and locomotion resumes thereafter. Mortality rate through these procedures is very rare and will be less than 1%.

Although the animals will be regularly monitored, weight loss has not been an issue with these procedures.

X-ray scanning. There is the risk that radiation doses during a micro-CT X-ray examination may produce adverse effects such as delayed growth or repair. However, doses of x-rays and duration of scans are way below any threshold that will cause problems to the animal. These scans are usually in the duration of 2- 5 minutes.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities are moderate as the animals will be undergoing a surgical procedure.

In the skin implant model, the severity can be mild as only an injection of biomaterial is added under the skin. For larger biomaterial implants the animals will be anesthetised and a skin incision made, implant inserted under the skin and sutured. Animals will be given pain relief post-surgery. Therefore, this procedure will have a moderate severity. We envisage about 60% will be moderate and about 40% will be mild.

For the bone defect models in the other protocols, we estimate the severity to be moderate and that 100% of the animal will experience this due to the surgical procedure. Again, these animals will be given pain relief post-surgery.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Initial bone tissue engineering research work and biocompatibility will utilise all relevant in vitro methods at our disposal. However, the clinical relevance of the approaches proposed, and the quality and quantity of bone tissue formed can only be assessed by in vivo studies in relevant animal models. In vitro approaches cannot, to date, mimic the complex physiological in vivo microenvironment involved in tissue repair. Thus, this proposal necessitates the use, in part, of animals to test the ability to generate new bone using the bone tissue engineering principles outlined. It is important to stress that initial in vitro studies are undertaken to evaluate and optimise the growth of human bone cells and skeletal stem cell populations on the scaffolds under examination. Where possible we will utilise our organotypic/organ culture in vitro models as a replacement to in vivo models to examine the ability of our regenerative strategies to repair skeletal defects. However, to address the efficacy of biomaterials in skeletal repair that require an understanding of its effects on integration, inflammation and blood vessel formation, then in vivo skeletal models will need to be used.

Which non-animal alternatives did you consider for use in this project?

Using laboratory cell culture experiments we are gaining as much information as possible to understand the functions and toxicities of new biomaterials being developed. In addition, we are using 3 dimensional bone tissue known as organoids to study the interaction of the many cell types that create fully functional bone tissue. We are using this data to create biomaterials with functioning coatings which can mimic the bone growth processes.

In other studies conducted in our group Artificial Intelligence (AI) modelling is being developed to test biomaterials implanted into the developing chick membrane, so that this technique can be used to predict and determine how a new material will act in producing blood vessels and new bone tissue.
However, there are limitations at the moment as this AI modelling is based on data provided from real studies. Once enough data has been accumulated for AI, this can, in principle, be applied to the animal models used in these protocols when researching new biomaterials. As AI modelling obtains a better understanding of the outcomes and mechanisms of these materials in the aforementioned bone models, the number of animals required will eventually decrease. With continual learning of the models and how new therapies interact in repairing and rebuilding bone AI in the future will be one avenue to replace the use of animal models.

Previous human studies. There is a wealth of clinical literature providing data on the efficacy of biomaterials on implants in hip replacements and bone fracture repair and in bone fracture that will

provide us with guidelines of what works in the patient. In addition, there are number of clinical trials which have data on the dosing of growth factors and of skeletal stem cells which give insights into the positive outcomes and the negative outcomes. We'll use this information to improve our biomaterials and avoid the negative consequences that have already been discovered in these clinical trials.

Why were they not suitable?

The above strategies are suitable and we do use them to screen, in part, the many biomaterials that are developed. We only resort to animal studies due to the complex factors that are involved in regeneration and repairing of bones. The generation of new bone requires multiple steps and the interplay of many different bone factors (inflammation, skeletal stem cells, blood vessel cells, collagen structures, calcium and phosphate (for hardening the new tissue), mechanical stimulation and cells that re-shape the new bone in a 3D environment to fit the defect bone exactly (without scarring) in a co-ordinated manner.

This interaction cannot be consistently or correctly reproduced under tissue culture conditions to meet the criteria expected to inform a clinical translation. In addition, in vitro model systems make it difficult to detect unexpected toxicities and observe the integration and degradation of materials over time.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used our current and past experimental data to inform us of an estimation of the number of animals required per group and the number of control and test groups to give statistically valid experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In the design phase we have used organ culture and chick eggs to reduce intra-group variability of biomaterials and by using this model and the results obtained has allowed us to reduce progressive experiments in mice models. This screening has reduced approximately 60% the regenerative biomaterials being taken forward for testing in animal studies.

We have designed experiments using the fewest animals consistent with obtaining statistically valid results as determined from our power calculations. For example, multiple small implants, up to six, can be assessed in one animal. This significantly reduces the number of animals per group required in these studies by 50%. Using the NC3R's Experimental Design Assistant we have calculated the

minimum number of animals required for a determined amount of new bone tissue formation in the test implant material comparison to control (no implants) or control (implanted biomaterials without stimulatory factors). With refined techniques and multiple imaging of the same animal over time, we can confidently obtain significant result outcomes with 4 animals per group.

In certain cases, mathematical modelling may be used to predict the release of growth factors from biomaterials under certain biological conditions, allowing us to determine how many animals we can exclude while still achieving a meaningful outcome in the experiments. In addition, multiple scanning and imaging of the animal bone implant or bone defect site at different time points reduces the number of animals required in these type of studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Rigorous laboratory testing will be performed on the newly constructed biomaterials before any candidates will be put forward for the skin implant experiments. In Stop/go scenario (Fig.1) only positive biomaterials in these protocols will advance to the bone defect models in the mice and rats. Furthermore, the best candidates in the rodent bone repair animals will be scaled up and used in a larger animal model.

Multiple analysis will be undertaken on biomaterial samples implanted into the animals, this will include X-ray and microscope imaging, blood vessel scoring, biochemical and molecular analysis, and histology. The multiple data that can be derived from one sample will optimise and minimise the number of animals to be used in a single study. In addition, real time optical/microscope imaging will significantly reduce the number of time points required to assess bone formation within the implanted biomaterial. This will further reduce the number of animals required for one study involving multiple time point assessments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used in the majority of the investigations in this project license as they are the best characterised and standardised species appropriate for this research. The microinjection model and the subcutaneous implant model will allow for the assessment of the localisation of the biomaterials to the skeletal site and new bone formation of the implanted samples respectively. This procedure will cause the least pain, suffering and lasting harm to the animals compared to the bone defect model protocols. However, this is a non-skeletal site, therefore, to properly establish the efficacy of these

materials, we must implant them into a bone location and see if they can combine and rebuild the bone to its former state.

Technically the methods refined from previous animal licenses are;

Surgery is performed early in the week allowing for more detailed observations post-surgery.

Staggered surgery procedures throughout the week so that there is plenty of pre and post-surgical time to assess the welfare of the animals undergoing the surgical procedures and also to have the optimal time for the preparation of the biomaterials to implant.

Pre & post analgesia given for the bone defect surgery and implantation.

In the first few days after surgery, tunnels, housing in the cages will be removed to reduce the potential risk of affecting the healing at the surgical site of the animal. Also, non tangling nesting materials will be used in the initial days after surgery.

Food will be placed on the bedding/floor of the cages initially in the first couple of days after surgery in the limb defect models to minimise increased load bearing and rotation on the operated femur.

With the calvarial defect model soft food will be administered as eating hard food pellets could cause some pain and discomfort in the cranial defect site

Finally, to address the clinical problem of large areas of bone loss failing to mend, the rabbit model is a well-characterized model for evaluating large biomaterials to repair this bone deficiency, allowing for the possibility, if successful, of transferring these therapies to the clinic.

Why can’t you use animals that are less sentient?

The development of new bone is incredibly complex involving factors and cells from the blood, the bone marrow, inflammatory cells, minerals and mechanical forces. In addition, bone fractures takes a considerable time to successfully repair.

Unfortunately, we are still not at a stage to incorporate all these components in the laboratory therefore these animal models are required to help us understand and improve the development of materials to aid in bone growth and repair. We can mimic some of components in the laboratory, and this has led us to refine the materials and cells that we will be used in these studies.

There is a chick egg embryo model that we use to determine the efficacy of these biomaterials in generating bone. This model is a less-invasive in vivo model allowing for the assessment and function of these biomaterials in a living organism. However, these are short term models (max. 10 days) whereas bone repair can take months to repair in vivo. In about 90% of our studies we use this model to test the biocompatibility and efficacy of new materials, gels, cells, growth factors before consideration in using in these in mice/rat models. Only clinically approved materials will be considered to be used directly in the mice/rat studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We shall continue working to minimize welfare costs for the animals in the following ways:

- Reviewing the need for these experiments to be undertaken. Weighing up the balance of potential successful outcome of undertaking the experiment and the level of harm/distress the animal will endure.

- We will ask for feedback from technicians and welfare officers in the Biomedical Research Facility and their valuable day-to-day knowledge to revise protocols to improve the animals' experience.

- Prior to the surgical procedures, researchers will familiarise and handle the animals on a regular basis to reduce stress for both the animal and the user.

- General best practise guidance for injection, blood sampling and aseptic techniques will be followed.

- Animals are closely monitored for several days after implantation under the skin or after the bone defect surgery, with continued pain relief given over 72 hrs in the first instance and further pain relief administered if signs of pain persists in the animal. Wounds will be carefully monitored to ensure that sutures have not loosened or come off or there are any signs of infection.

- Animal weight is frequently monitored for the duration of the studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the ARRIVE guidelines which provide a 20-part checklist of the minimum information required to be reported by groups using animals in research. ARRIVE

guidelines are essential to help overcome issues in science such as reproducibility, reducing bias and the correct use of statistical methods of analysis.

In addition, we will follow and consult the Norecopa (Norway's National Consensus Platform for the advancement of "the 3 Rs" (Replacement, Reduction, Refinement)) in connection with animal experiments database platform and PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for better science experiments using animals to ensure that we are using the best defined models for our work in the investigations of biomaterials for skeletal biology.

All surgeries will follow the LASA (2010) guiding principles for preparing for and undertaking aseptic surgery. The standard operating procedures (SOPs) and risk assessments for these surgical procedures will be evaluated annually and amended in accordance with published updated aseptic surgical practises and guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will consult the NC3Rs website to identify any changes that will be relevant to the workings of this model. In addition, we will follow the latest findings from the Laboratory Animal Science Association (LASA) and the PREPARE guidelines from Norecopa in better planning for research involving animals to prepare for better science and advance the 3Rs. Any changes will be implemented directly through the experimental design, and if necessary, through a project licence amendment.

In addition, we follow the latest publications on using these models and identify any new methods that reduce, replace or refine the skin implant and bone defect models. If applicable to this model in improving the 3R's we will request amendments from the Home Office to adjust the techniques/methods required and training or notification of relevant staff in updated techniques.

Continual training will be undertaken to ensure that users will be proficient in running these studies particularly on the aftercare post- surgery.

Attendance at University animal users meetings and/or discussion of University animal users meeting slides at research group lab meetings.

We will liaise with other experienced groups in in vivo research both nationally and internationally who use these models to get advice on updates on best practise for anaesthesia/analgesia, husbandry and surgical procedures similar to the ones we will be undertaking in this project.

202. Brain Mechanisms Linking Early Life Adversity to Alcohol Addiction

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Alcohol addiction, Early life adversity, Adolescence, Neuroscience, Mental health

Animal types	Life stages
Rats	neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to define how early life adversity and adolescent alcohol consumption alter brain function to increase the likelihood of alcohol addiction in adulthood. The secondary aim is to test drugs with the potential to treat alcohol addiction.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Alcohol use disorders (AUDs) affect over 100 million people worldwide, with alcohol use accounting for 4.2% of global disability adjusted life years (DALYs). In the UK, alcohol misuse is estimated to effect 1- 2% of people; it is the biggest risk factor for death, ill-health and disability among 15-49 year-olds, and the fifth biggest risk factor across all

ages. Understanding the triggers for alcohol dependence is therefore a vital first step in finding ways to mitigate this risk.

Experience of adversity and/or alcohol consumption during the critical developmental windows of early life and adolescence are major risk factors, culminating in increased rates and severity of AUD. Whilst the understanding of how specific brain regions contribute to alcohol use disorders continues to improve, there is no working model of the brain changes linking early life adversity and adolescent experiences to the changes occurring at the level of neuronal circuits.

The association between trauma and substance use disorders has been recognised by the UK Department of Health, with clinical services required to use a trauma-informed model of addiction care. Adverse Childhood Events (ACE) specifically linked to alcohol use include physical abuse, sexual abuse, having a mentally ill household member, substance abuse in the home, and parental discord or divorce. In epidemiological studies, ACE predict earlier onset of alcohol use and increased levels of alcohol consumption. All forms of childhood trauma act as risk factors for adolescent binge drinking, with earlier alcohol consumption potentially lying on the causal pathway between trauma and alcohol use disorders.

Adolescence (defined by the World Health Organisation as spanning ages 10-19 years) is a critical window for the development of AUDs due to the vulnerability the developing adolescent brain. AUDs commonly emerge during late adolescence and early adulthood, with a median age of onset for alcohol abuse of 21 and alcohol dependence of 23. Cohort studies confirm that adolescent alcohol use has both immediate and longer-term effects on the development of AUDs, increasing the risk of alcohol addiction across the lifespan, most likely by triggering enduring changes in brain physiology and function.

Understanding the mechanisms causally linking early life adversity and adolescent alcohol use on the development of AUD in adulthood has vital implications for health and social policy. This programme of preclinical work will be integrated with a complementary programme of epidemiological research on human data using the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort, and will include collaboration with a drug development commercial partner. For example, one pathway that may explain how early life adversity and adolescent alcohol use lead to AUD in adulthood is through long term changes in gene expression as a result of epigenetic modification. Exposure to early life adversity and adolescent alcohol use both trigger long-term epigenetic changes, with markers of early life adversity inducing conserved epigenetic signatures in both human and rat brain tissue. Brain gene regulation therefore provides scope for translation and model validation between the preclinical and epidemiological studies.

Despite the contributions of early life adversity to the pathogenesis of addiction, none of the pharmacological treatments in clinical use are based on the treatment of traumatic experiences from early life. Current addiction treatments have limited efficacy, with most patients that achieve abstinence either alone, or with treatment, experiencing subsequent relapse. An improved understanding of the pathways from early life adversity to addiction in adulthood is a necessary step towards development of drugs targeting these pathways. The data obtained through this experimental work will inform cellular and neural circuit-resolution models of alcohol addiction, that can in turn be translated and validated in human brain imaging studies. Defining the neural signatures underlying alcohol addiction enables translational assays for new treatment approaches to addiction. Once models of

AUD are established, both current and prospective treatments for AUD will be tested to determine how they alter the function of neural circuits within the scope of these models.
What outputs do you think you will see at the end of this project?

The study is expected to generate the following outputs:

1. A refined rat model of alcohol addiction combining early life adversity with operant drug seeking

2. Behavioural fingerprints of alcohol addiction in rats, including measures of activity, anxiety and sleep.

3. Mapping the neural circuitry linking early life adversity, adolescent alcohol exposure, and AUDs in later life.

4. An epigenetic profile of early life adversity, adolescent drug use, and adulthood addiction, informing translation of phenotypes between preclinical and human studies.

5. A computational model of cortico-limbic and cortico-striatal circuits in alcohol addiction based on electrophysiological data and, where possible, integration with measurements of neurotransmitter activity.

6. Rational design and testing of novel therapeutics based on the mechanistic insights gained from the project.

Data generated during this project will be shared in the form of publications (open access preprints and peer reviewed journal articles), with non-proprietary behavioural, epigenetic and neurophysiology data made open access.

Who or what will benefit from these outputs, and how?

Within the 5 years of this project, primary beneficiaries will be preclinical and clinical researchers working on the neurobiology of addiction. The work defining the neural circuits linking early life adversity to alcohol addiction is designed to help others refine their own studies. For example, the development of a remotely-monitored, operant alcohol delivery system for home-cage behaviours will have important 3Rs benefits for the field. Our commercial collaborators will also benefit from the integration of the refined rat model of AUD into their drug discovery pipelines.

Ultimately, it is to be expected that the outputs of this work will benefit patients with AUD and other forms of addiction, primarily through the development of treatments based on an understanding of the neural pathways and molecular triggers involved. In addition, a better understanding of the mechanisms linking early life adversity, adolescent experience and AUD can be expected to benefit the public at large by informing the development of public health policies to prevent the development of addiction.

How will you look to maximise the outputs of this work?

Sharing of experimental design through protocol pre-registration where appropriate.

Discussion of approach and results at public events (e.g. Neuroscience Festivals) and national and international academic conferences.

Timely publication of results as preprints and peer-reviewed articles.

Collaboration with industrial collaborators on treatment development.

Liaison with clinical colleagues to optimise translation and patient benefit.

Species and numbers of animals expected to be used

- Rats: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rats are the most suitable species for this study as they provide the least sentient established model of alcohol use disorder (AUD). Rats have been used successfully to demonstrate most behavioural components of human AUD experimentally, including the transition from habitual to compulsive alcohol-seeking and the effects of early life adversity in increasing AUD. Rat models of AUD have also
shown predictive validity for assessing drugs aimed at modulating drinking in humans, with some novel compounds, shown to be effective in rats, progressing to early phase human clinical trials. Due to the importance of early life experience in the development of addiction and the long-term nature of AUD rats may be used from birth through to adulthood in these studies.

Typically, what will be done to an animal used in your project?

Rat pups will be reared under a maternal separation model of early life adversity, involving separating the pups from their mother 3 hours per day during the pre-weaning period. Following weaning, the rats will be provided with free access to alcohol in their home cage via an operant conditioning system (nose poking to deliver liquid) to establish their alcohol dependence. Thereafter, the young adult rats will undergo a single recovery surgery during which they may be injected with a virus expressing optogenetic reporters and implanted with recording electrodes and optic fibres.

Following recovery from surgery and transition into adulthood, animals will have access to alcohol and water in the home cage via an operant system, with the pattern of access recreating the human cycle of binge-withdrawal seen in AUD. Brain activity will be recorded during seeking and drinking of alcohol, with behaviours monitored by video for scoring of addiction-associated behaviour and sleep/activity tracking. Anxiety levels will be determined using non-invasive behavioural tests such as the elevated plus maze.

At the end of the experiment (typically 4-6 months old) animals will be killed by either perfusion fixation under terminal anaesthesia, or a schedule 1 method, and the brain harvested for histological and epigenetic analysis. Behavioural assessments, electrophysiological recording and blood sampling will be undertaken at key time points during the course of the experiments.

What are the expected impacts and/or adverse effects for the animals during your project?

Prior to weaning the dam and her pups are expected to experience mild distress as a result of maternal separation. The consumption of alcohol by weaned animals may result in mild behavioural changes. All animals are expected to suffer mild post-operative pain during the first day or two following surgery.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for all animals in this study is moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Whilst epidemiological and epigenetic studies in humans have clearly demonstrated the link between early life experience and alcohol use disorder (AUD) investigating the neurophysiological basis of this addiction in humans is not feasible owing to the invasive nature of the procedures involved. Since early life stresses can only be experienced by an animal with a highly developed brain, it is not possible to conduct these studies using non-living or cell or organ culture systems. Consequently, there is no alternative to the use of living animals for these studies.

Which non-animal alternatives did you consider for use in this project?

1. Human subjects

2. Non-regulated species e.g. Drosophila

3. Electrophysiology in terminally anaesthetised animals

4. In silico computational modelling

Why were they not suitable?

Human Subjects: The location of the neurocircuits involved in AUD lie in deep brain structures, such as the basolateral amygdala and striatum, which are beyond the temporal or spatial resolution of current non-invasive imaging and electrophysiological recording systems used for monitoring human brain activity. The mechanistic approach required to address the aims of the outlined studies cannot be applied to humans for both practical

and ethical reasons. Nevertheless, the programme of work will benefit from epidemiological and epigenetic studies conducted in parallel on human data collected as part of the Avon Longitudinal Study of Parents and Children (ALSPAC).

Non-regulated species e.g. Drosophila: Invertebrate nervous systems offer limited insight into mammalian neural circuits involved in cognition and disease. Consequently, a working model relevant to humans AUD is not achievable due to the differences in brain anatomy and physiology.

Electrophysiology in terminally anaesthetised animals: This can offer important insights into functional connectivity, however, general anaesthetics by definition massively alter brain state, and are likely to obscure the effects of the AUD models and anaesthesia precludes combination with behavioural measures. Where possible work in freely behaving animals will be combined with in vitro studies of neural activity (e.g. in brain slices) and with computational models of brain activity.

In silico computational modelling: The use of modelling requires simplified approximations and cannot be used to generate the initial data for this project. Computational models will be used during the analysis of animal data, and will allow specific structural hypotheses to be tested without further animal studies. These in silico models may form the basis of future translation into human studies using lower resolution brain imaging.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals has been estimated based on a 15 year track record of related experimental work describing changes in brain network activity across different experimental states, plus from related literature and advice from industrial collaborators. The studies under this project will use the minimum number of animals that are anticipated to yield robust and reliable results and meet Objectives 1-5. We expect numbers to be distributed as follows:

Objective 1, To refine a rat model of alcohol use disorder based on pre-weaning maternal separation and operant alcohol drinking: 40 rats

Objective 2, To assess translational phenotypes (for example anxiety, sleep) that may lie on causal pathways between early life adversity and alcohol consumption in adulthood: 40 rats

Objective 3, To measure brain activity and neurotransmitter signalling in rats with alcohol addiction: 40 rats

Objective 4: To test the effects of potential pharmacological treatments of alcohol use disorder: 80 rats

Objective 5: To quantify brain/blood epigenetic changes seen in early life adversity and established alcohol addiction: this will use tissue harvested from rats used in Objectives 1-4

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of animals used is based on analysis of previous data collected by the team, cross- checked with the NC3Rs Experimental Design tool and using power calculations and a statistical framework agreed with the institutional statistics clinic. The design has been developed to collect multimodal data from a single set of animals, rather than running separate behavioural and electrophysiological studies in multiple groups.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The methods we use are highly efficient at gathering large volumes of data from individual animals using a within-subject design, meaning the number of individual animals used is kept to the absolute minimum required to allow statistically robust conclusions. Each experiment is mapped out from the outset, working with facility staff to assign each animal 'Study Plan' to optimise its contributions to the

dataset, typically addressing multiple objectives during a single procedure (for example by recording during both behaviour and sleep). Breeding to generate pups for maternal separation is agreed with and overseen by experienced technical staff to minimise production of unused animals. Each animal will generate behavioural measures, measures of brain activity, brain tissue for epigenetic analyses and post mortem samples (e.g. of gut for microbiome analyses) for sharing with collaborators.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this project we will be developing and refining a homecage operant approach to alcohol exposure in rat. This has the benefit of reducing intervention (e.g. negating stress from oral dosing), as animals do not need to be removed from the home cage for operant alcohol administration sessions. The approach also more accurately recapitulates human behaviour. The homecage system includes automatic delivery and measurement of liquid following nose-pokes, with sensors also capturing approach behaviours via video records. This allows the collection of continuous data of alcohol seeking and consumption, allowing high-resolution characterisation of behaviour. The use of an operant approach to alcohol access also allows for the separation of the neural signatures of alcohol-related cues, and alcohol consumption, which is of relevance with regards the importance of cues in perpetuating AUD and precipitating relapse to AUD in humans.

To model the effects of early-life adversity on alcohol consumption, we will use maternal separation of pre-weaning pups. Separating pups from their mother for 3 hours per day is a well-characterised, consistent model of adversity, allowing us to build on an extensive literature while avoiding additional distress inherent in longer separation paradigms.

The data analysis programs and computational methods used in this project are state-of-the-art, and analogous to those required to analyse human EEG and brain imaging data, and are made freely available online.

Why can't you use animals that are less sentient?

Invertebrate brain development - for example in flies - is not sufficiently similar to human brain development to enable invertebrate models of mechanistic links between early life experience and adult alcohol use. We are aware of emergent models of addiction in less sentient vertebrates, for example zebrafish. However, fish development is very different from that of mammals, and operant drug seeking is difficult to implement underwater. It is not currently possible to monitor fish brain activity during free behaviour.

There are published protocols for (a) maternal separation and (b) alcohol drinking in mice, plus some mouse lines prone to higher alcohol consumption. However, these protocols are less robustly established in mouse than in rat, with the literature revealing mixed results. This is particularly true for patterns of alcohol seeking and drinking, with the intermittent access typical of human Alcohol Use Disorder not always leading to elevated drinking in mice. Chronic cranial implants in mice - particularly young mice - also have proportionally greater welfare implications, given that size and weight of implants must be limited.

In summary, rats therefore offer the optimal combination of established protocols, translational validity and compatibility with behavioural neurophysiology.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Using an operant model of alcohol consumption within the home cage presents significant refinements compared with commonly used models. Higher resolution data on drinking behaviour can be captured while simultaneously reducing intervention and handling of the animals. Operant access also allows the supply of alcohol to be tightly controlled through the computer controlled system preventing excess consumption that could lead to acute toxicity or overdose.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3Rs Experimental Design Assistant, ARRIVE 2.0 guidelines for design and reporting, local SOPs for handling, LAVA guidelines for aseptic surgery, comprehensive and contemporary review of published papers in the field.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive regular 3Rs newsletters from local and regional NC3Rs champions, and monitor advances in best practice by regularly attending conferences in the field, liaising with national and international collaborators, and through weekly journal clubs appraising

recent publications. We also maintain live collaborations with engineers and technology developers in the field, optimising opportunities to pilot and/or implement the most efficient and minimally invasive methods of neurophysiological data collection.

203. Role of Arp2/3 Isoforms in Mouse Development and Tissue Homeostasis

Project duration

5 years 0 months

- Project purpose
- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Actin cytoskeleton, Development, Immunology, Neurology

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how the cell's internal skeleton is regulated by specific protein complexes during tissue formation and maintenance, particularly in blood, brain and immune function.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Actin, one of the most abundant proteins of the cell, assembles into linear polymers to make up the actin skeleton of the cell. This internal actin skeleton provides the driving

force and structural support for the physical integrity of cells and a wide range of essential cellular processes such as cell migration and adhesion. The precise spatial and temporal regulation of the actin cytoskeleton is crucial during the development and throughout the lifetime of multi-cellular organisms. Consequently, dysregulation of the actin cytoskeleton impacts tissue and organ function including the brain and the immune system, and in the worst case, the development and lifespan of an organism. Studying how the Arp2/3 complex which is unique in the cell in promoting the formation of branched actin polymers to regulate the function of the actin cytoskeleton is therefore key to understanding human development and tissue maintenance to uncover the basis of a wide variety of human diseases such as hereditary ataxia and combined immunodeficiency .

What outputs do you think you will see at the end of this project?

The main object of our work program is to obtain a detailed understanding of the role of Arp2/3 complexes in regulating cellular processes controlling the development and function of organs and tissues. The outputs of our project will be mainly in the form of publications in peer-reviewed articles and presentations at scientific conferences. This project will create novel mouse strains, which will be available for the wider research community. It will also provide insights into the basis of human conditions and diseases such as neurodegeneration, inflammation, and immunodeficiency.

Who or what will benefit from these outputs, and how?

Emerging genetic evidence in humans has already established the importance of actin cytoskeleton and the Arp2/3 complex in human development and health. For example, loss of function mutations in human ARPC1B, the gene encoding one of the proteins within the Arp2/3 complex, leads to Wiskott- Aldrich syndrome-like symptoms including severe inflammation and immunodeficiency. Mutations in the genes encoding the other proteins of the Arp2/3 complex are also predicted to impact human health. Therefore, to fully understand the function of the actin cytoskeleton, we will need to study how it is regulated by the Arp2/3 complex in living animals.

In the short term, the new scientific insights obtained from our work will be beneficial for other scientists including developmental and cell biologists investigating how regulation of the actin cytoskeleton controls the form and function of cells and tissues during development and adult life. In the long-term, the phenotypes and molecular mechanisms we uncover will also benefit scientists and clinicians trying to understand the underlying basis for pathogenic and genetic conditions of the nervous and immune systems that lead to disease or dysfunction. Basic scientists and clinicians will also benefit from the mouse strains we will generate during this project.

How will you look to maximise the outputs of this work?

We will share our data, analysis tools, and resources with other scientists, using scientific communications and collaborations, repositories, and our lab webpage, which will reduce the number of replicated experiments in the field. We will disseminate our research by publishing results in peer- reviewed journals. We will also present our work to academic peers at scientific conferences and engage with public partners to disseminate our results. The mouse strains we generate will be shared with collaborators and the wider community (scientists and clinicians).

Species and numbers of animals expected to be used

- Mice: 28000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will investigate how Arp2/3 complexes regulate mammalian development and tissue maintenance, consequently, mice represent the best available animal model given the many similarities with human development and availability of genetic tools. As we are investigating the changes that occur during animal foetal development and throughout their lifetime, it is necessary to use embryonic, postnatal, adult, and aged mice.

Typically, what will be done to an animal used in your project?

The majority of our experiments will involve the generation and breeding of genetically altered mice. Embryos and animals may also receive antibiotics and other drugs such as Doxycycline, Tetracycline and Tamoxifen through injection or via their diet and water in order to induce gene deletion or activation. Animals will be tested for gait, cognitive development, and social behaviour to evaluate their neuronal function. To examine the changes in the actin cytoskeleton and the Arp2/3 complex during the normal aging processes, animals with no phenotypes may be kept for up to 16 months and animals with adverse effects for up to maximum 12 months, respectively. When needed animals will be housed in the Digital Ventilated Cage (DVC) for 24/7 monitoring and analysis of animal activity to detect any behavioural changes. Cell labelling reagents such as 2-deoxy-5-ethynyluridine (EdU) and Adeno-associated virus (AAV) may be introduced via injections prior to the humane killing at later time points. Blood may be withdrawn by microsampling (<1% total blood volume) every 7 days or at maximum
<15% total blood volume in 28 day intervals. In some cases, general anaesthesia and contrast imaging agents will be administrated prior to non-invasive imaging up to once a week but no more than 20 times during an animal's lifetime. Generally, administration of substances and behavioural tests will only cause transient discomfort and no lasting harm.

To investigate the role of the actin cytoskeleton in blood and immune cell function, we will immunologically challenge animals using immunity inducers such as egg white, induce colitis for 5 days using DSS or infect animals with intestinal pathogens as these procedures will induce conditions that are similar to that seen in humans with deficiencies in the Arp2/3 complex. The animals will be followed for a maximum of 6 months and may also receive antibiotic treatments before and during the procedures. These treatments will be performed once, after which animals are killed. These analyses will also involve the isolation of immune cells from genetically altered animals, which will then be transplanted into the host animals (commercially available immunodeficient mice or mice treated with whole-body irradiation beforehand). When required, these different procedures will be performed in combination.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of genetically modified animals we will generate are expected to display either no phenotypes or mild phenotypes, which do not impact the behaviour, gross appearance or welfare of the animal but are only evident at post-mortem after humane killing. In some cases, a limited number of animals will experience moderate phenotypes: these might include abnormal gait and loss of motor coordination after 3 months as a consequence of neurodegeneration and possibly laboured breathing due to cardiovascular dysfunction as well as limited weight loss, scruffy fur, and hunching in response to infection. Animals with motor coordination defects will be humanely killed before they display clinical signs such as reduced and/or misdirected movement, abdominal discomfort, hunched posture, or ruffled fur.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Based on our experience to date, we are expecting less than 45% of animals to exhibit moderate severity. All other animals will only have mild severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Laboratory-based experiments in the test tube (biochemistry) or in cells in a dish (cell biology) have greatly enhanced our molecular understanding of how the actin cytoskeleton regulates processes that control the form and function of cells. Such analysis may guide but will not fully uncover how the actin cytoskeleton controls mouse development and tissue maintenance. This is because these highly complex processes involve the dynamic interaction of multiple proteins, cell types and physiological systems, that need to work together and change over time. Currently, no cell-based system in a dish can fully replace or replicate all these factors. Therefore, to fully understand the function of the actin cytoskeleton and disease we will need to study how it is regulated in living animals. Our in vivo studies may also expose phenotypes that implicate Arp2/3 isoforms in various idiopathic diseases, thereby opening up new targets in disease treatments.

Which non-animal alternatives did you consider for use in this project?

We currently use a variety of biochemical assays with purified proteins as well as cell-based approaches including live-cell imaging to characterise and study the regulation of the actin cytoskeleton. The results from these non-animal-based experiments provide mechanistic and molecular insights into the function and regulation of the actin cytoskeleton. Importantly, this information as well as hypotheses generated from experiments in test tubes and cells on a dish helps us to reduce the number of animal-

based experiments by only focusing on the function of Arp2/3 complexes in controlling the actin cytoskeleton in specific tissues.

Why were they not suitable?

The isoform-specific Arp2/3 function cannot be investigated in non-animal organisms such as yeast and fruit flies as they do not have these Arp2/3 isoform genes. Biochemical and cell-based approaches are extremely powerful; however, they do not supplant the use of animals to understand the function of Arp2/3 isoforms in regulating the actin cytoskeleton. This is because cells in a dish fail to capture the full complexity of tissue organization which involves the complex and integrated interaction of many different cell types and the communication between different organs and systems (e.g., immune, hormonal, nervous etc) of the animal. We will consider organoid cell culture differentiated from ESC and patient iPSC as non-animal alternatives. Those cells can self-organise tissue-like structures that will help us to refine the design of our animal experiments and reduce animal use.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our animal use projections are based on three main factors:

1. The number of genetically modified lines we are currently maintaining.

2. The number of genetically modified lines we are planning to create.

3. The complexity of the crossing schemes to generate experimental animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use several strategies to limit the number of mice we use including:

1. Where possible we use both sexes for all experiments and always maximise tissue usage and the amount of data we get from each mouse.

2. We will use the NC3R's Experimental Design Assistant or similar software such as G*POWER to perform power calculations for each experiment.

3. We follow PREPARE guidelines and the checklist.

4. Avoid duplication of any work that has already been published by other groups, after extensive literature searches of published work on Pubmed and the BioRxiv pre-print server.

5. Where possible use results from in vitro cell culture models and ex vivo tissue culture models to generate hypotheses to guide our animal-based experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?
In addition to the measures outlined above, we ensure that all lab members minimise animal numbers through the following approaches.

1. Being fully aware of all aspects related to the 3Rs and using available online resources such as Experimental Design Assistant pages.

2. Carefully review our mating strategies to ensure we generate the maximum number of animals with the desired genotypes with the minimal number of breeding steps and avoid overbreeding. Where possible, we will set up pairs of homozygous and heterozygous to produce double the number of homozygous offspring when compared to the mating of heterozygous pairs.

3. Regularly review our mouse colony and where possible archive strains by freezing sperm and embryos.

4. When available, we will use in-house strains from our animal facility to promote efficient breeding. Most of our strains except for experimental strains will be maintained by intermittent breeding with in-

house wild-type mice to reduce animal use (generally breeding twice a year rather than constant breeding; NC3Rs guidance for breeding and colony management).

5. Use the minimal number of mice needed for statistical power when testing an experimental hypothesis based upon pilot experiments to inform on the numbers required. Also, use published and unpublished data in the lab to optimise the animal number required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is chosen because it is the least sentient mammal that expresses all eight Arp2/3 isoforms that is suitable for efficient genetic modifications. Mouse embryonic development, normal biology, and behaviour are well described, and there are well-established husbandry techniques for this species, including efficient analgesic methods. Many useful tissue-specific or inducible Cre mouse lines already exist that can be crossed with our Arp2/3 isoform mutants, obviating the need to create more transgenic Cre lines. The mouse is also suited to study the physiological regulation of the actin cytoskeleton by the Arp2/3 complex because the biology and pathophysiology of mice are relevant to

humans. Mice will therefore serve as an accurate animal model for a wide range of human conditions and pathologies. For example, disease-causing mutations, subsequent pathogenesis, and subtypes of neurodegenerative diseases including Parkinson's disease and spinocerebellar ataxia are well conserved in humans and mice. Most of our studies will be performed on dissected tissue and ex vivo tissue culture from humanely killed animals.

To investigate the function of the actin cytoskeleton in blood and immune cells (i.e. haematological and immunological function), we will use the following methods by injection or intake through food and drinking water: exposure to inducing agents (e.g. OVA and LPS) that are expected to cause no or mild harm regardless of the genotype background, bacterial infections that may cause gastrointestinal discomfort in some mutant strains and DSS-induced colitis model. These models and procedures will allow us to uncover insights into human disease as they induce conditions that are similar to those seen in humans with deficiencies in the Arp2/3 complex. As these methods/approaches are well described, it is straightforward for us to know/use the lowest concentration of agents or pathogens to apply to our mice to ensure their well-being while still achieving our goals.

To investigate the function of the actin cytoskeleton in the nervous system (brain), we will use a genetic approach to ablate Arp2/3 isoforms. In some cases, our genetically altered mice develop abnormal behaviour coincident with progressive loss of neurones and/or their function. These phenotypes, which result in reduced coordination replicate the normal aging processes rather than traumatic brain injuries. To further minimise suffering and harm to animals, we will modify genes only in neuronal cell types and/or temporally rather than in the whole animal and permanently where possible. We will assess animal gait, cognitive development, and social behaviour by the established behavioural tests that do not lead to pain or distress to ensure animal well-being during analyses.

Why can't you use animals that are less sentient?

The major development of the nervous and immune systems happens after birth even though those systems first appear during foetal development. Given this, we cannot use embryos to study the physiological regulation of actin cytoskeleton during tissue development and maintenance, with emphasis on blood, brain, and immune cells. Where possible, ex vivo tissue culture from animals in the early stage of life will be used to uncover phenotypes to reduce animal numbers and suffering. The mouse rather than another species such as fruit flies or worms is used because they are the least sentient mammal that expresses the eight different protein complexes that we are studying.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals with neurological phenotypes and impaired movement by genetic alteration or aging may result in a reduction in feeding and water intake. Given this possibility, in addition to weekly weighing, these animals will be offered increased monitoring according to a scoring sheet that includes the following parameters: activity; posture; movement/gait; coat condition; breathing, and dehydration (as per Wilkinson et al., 2019; DOI:10.1177/0023677219865291). Experimental animals are weighed weekly and extensively monitored for pre-/post-procedure health and good welfare on a daily basis according to The Mouse Grimace Scale available at NC3Rs. Animals will be offered wet food as well as additional bedding and cage enrichment if necessary. Animals exhibiting detrimental phenotypes that are not common in our project will be immediately humanely

killed. Pilot studies guided by previous observations on DSS-induced models in the establishment will be performed to determine the minimal dose of DSS required to induce colitis in our mice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the NC3Rs website for the general principles of blood sampling and substance injection. We will follow PREPARE guidelines for experimental design, sample size, and ARRIVE for reporting our results. In general, we will follow guidance from “Refining procedures for the administration of substances” (Morton et al., 2001; DOI:10.1258/0023677011911).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed of best practices, 3Rs and any implementation in animal handling through newsletters and communications on slack regularly published from our animal facility. We will seek further information from the NC3Rs website and our NC3Rs programme manager. This includes by finding NC3Rs projects relevant to our studies particularly in the brain and immunological research at NC3Rs our portfolio page as well as attending 3R Online-Seminar series “Alternatives to animal use in research and education – Refine, Reduce & Replace“, provided by Berlin-Brandenburg research platform BB3R.

204. Refining Strategies for the Treatment Of Human Cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

cancer, therapy, cancer tissue interaction, cancer treatment resistance

Animal types	Life stages
Mice	adult, juvenile, pregnant, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to test new ways that could improve the treatment of cancers compared to current treatment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

While progress has been made in the treatment of cancer there are clearly defined problems that urgently need solutions. For example, cancer despite initial response to a treatment regrow in a resistant form, or treatment is not effective at the outset in some patients.

New information indicates that molecular characteristics of cancer determine resistance and response in individual patients. Procedures that target such molecular characteristics are a highly promising route and may improve the likelihood of a patient surviving.

Under this programme licence, we will seek to prove the validity of molecularly tailored strategies which we continue to discover in laboratory-based research that uses cancer cell-based methods.

Using animal-based models of human cancer to document the ultimate functioning of treatment is a critical requirement for such treatments to become accepted for the treatment of human patients with cancer.

What outputs do you think you will see at the end of this project?

Expected “outputs” from the project are novel anticancer treatments, that is treatments that yield improved response in patients compared to currently used treatments.

A further output is the development of improved cancer models that are kinder than the currently used murine models and more faithfully reflect the presentation of disease in human patients.

In all cases, results using simple laboratory methods are a first step in discovering such new treatments. However, demonstrating that they work in an animal with the relevant disease is a critical step before such treatments can be adopted for use in human patients.

Who or what will benefit from these outputs, and how?

The “benefit” of these “outputs” should be seen within some years from when results are obtained. These benefits will initially come from so-called “human trials” where patients suffering from cancers where current treatments do not work are treated. If these trials show the treatment works, then it can be developed to be available to all relevant patients.

How will you look to maximise the outputs of this work?

We will publicize outcomes in scientific journals as a first step by which new results are communicated to the scientific community and become accepted. This is most important when results are positive.
However, we will also make sure that unsuccessful attempts are publicized because this will hopefully prevent others from using animals to test the same unsuccessful idea. We will particularly make sure that the information reaches doctors and the pharmaceutical industry and patients affected by disease. A highly effective route to publicize early results is to speak about them at scientific conferences and to post results early on the internet. All our work involves clinical doctors, which ensures the work we do will be tailored to help those patients for which truly there currently is insufficient treatment.

Species and numbers of animals expected to be used

- Mice: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The work under this project licence application will use adult mice. The use of mice to study cancer treatment is considered the gold standard and a necessary step before such treatments can be used in human patients.

Reasons, why the use of mice is considered a necessary and important step, are that mice are sufficiently similar to humans and also because special mice have been developed that can carry cancer isolated from human patients. These two special characteristics make mice a prime choice for the testing of cancer treatments.

Typically, what will be done to an animal used in your project?

Roughly one-third of the mice used will be used as breeding stock. These mice will carry alterations in their DNA (i.e., they are genetically modified) such that they do not reject human cancers when these are implanted. A portion of mice may develop cancer spontaneously.

In one-thirds of animals, small amounts of cancer cells will be injected under the skin, into the fatty tissue and in rare situations into the bloodstream of mice. In some animals (maximally 30 per year) a small incision will be required with surgical wound closure for the insertion of tumour fragments. For any of these procedures, mice will be put to sleep and mice be given medication that prevents any pain to be experienced.

Medicines, including homone treatment and antibiotics, will be given to the mice (rouhgly 20% overall), with the intent to treat or support the cancers they carry. As a rule, different groups will be treated in different ways to compare treatments. In the majority of cases, medicine will be given by mouth (orally). Wherever possible this will involve voluntary take, where medicines are mixed with foods that mice like to eat. Rarely, where medicines may taste bad even if they are mixed with tasty food and the mice do not take them, the substances may be given via a tube leading to the stomach (orogastric gavage) or by injection. In these cases, mice are not expected to suffer more than the short time that it takes to give the medicine. It is likely that medicines need to be given daily over several weeks in order to be effective. The medicines that will be used will have been made for use in humans, and because of this the amount to be used and their side effects will be known and there may be ways to ease these if they arise.

Tumour imaging, for example using magnetic resonance (MNR) or copmuterised tomography (CT) customary used in human patients will be needed to monitor tumour treatment response in a fraction of mice (10% of mice receiving treatment). Mice will be anaesthetized for the time imaging is undertaken

A small number of mice (less than 5%) may be treated using radiotherapy, applying doses aligned with those that may be received by patients. Mice will be anaesthetized for the duration that radiation therapy is given.

What are the expected impacts and/or adverse effects for the animals during your project?

A portion of mice used in this work will carry cancer. These mice will be observed closely, and steps will be taken so that they do not suffer, including providing pain medicines where this is required. Mice may experience mild pain or discomfort from needle insertion or insertion of a stomach tube to feed them medicines, but this is expected to be only transient.

In rare cases (10 % of treated), mice may grow more tired and/or lose appetite due to the disease they carry or the medicines or radiation therapy that they receive. Mice will be closely observed, and their weight monitored to make sure any weight loss that may harm the mice is detected early. As soon as any problems are identified mice will receive special care. This will include giving them soft and especially tasty food that they can reach easily from their nest. If weight loss exceeds limits above 15% that cannot be corrected using the above methods mice will be humanely put to death.

Mice receiving radiation treatment may develop mild skin reactions (30% of mice receiving radiation treatment), rarely (5% of mice receiving radiation treatment) may experience transient shortness of breath.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect that one-third of the mice will not experience any discomfort or pain as they are used in breeding. The other two-thirds of the mice may experience short episodes of moderate severity where mice feel quite unwell. Where moderate severity is observed that cannot be reversed or treated the animals will be humanly put to death.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Work under this licence would be informed by work in cell-based disease models. Such models include complex patient-derived cancer cells models and "organotypic models" where cancer tissue is grown within its normal tissue context. We are actively involved in developing such advanced models using tissue engineering technology. While evidence from such non-animal alternatives is not considered sufficient as preclinical evidence, positive results from non-animal alternatives will be a requirement for us to undertake animal-based work. As a principle, we are committed to exploiting to the full any alternatives that can replace, refine or reduce the use of animal-based procedures.

Which non-animal alternatives did you consider for use in this project?
Work under this licence would be informed by work in cell-based disease models. Such models include patient-derived cancer cells including organoids and "organotypic models"

where cancer tissue is growing within its normal tissue context. We are actively involved in developing such models, where we seek to build the normal tissue context of the cancers we study using tissue engineering technology. While currently evidence from such organotypic models is not considered sufficient to replace organismal level preclinical evidence, such models constitute important intermediates by providing additional support prior to engaging in animal-based work or by uncovering limitations that then would obviate animal experiments. As a routine, any ex vivo developments that can replace, refine or reduce the use of animal-based procedures will always be exploited to seek ex vivo proof of concept.

Why were they not suitable?

The response of cancer to therapeutics is affected by organismal responses that cannot currently be faithfully modelled using tissue-level alternatives. Relevant responses that cannot be adequately reproduced are organismal level side effects of treatment and also the interaction of diseased tissue with adjacent or distant tissues, which is recognised to influence the response to treatment.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers of mice needed are estimated based on previous studies and power calculations based on these. The number provided breaks down as follows. Mathematical calculations informed by pilot data will be used to estimate the minimal number of animals required for a given study.

An estimated one third of the mice (300 over the life of the PPL) are required solely for the purpose of breeding, with aim to safeguard the respective mouse strains and to produce animals needed for the different protocols under this licence. Breeding will be managed to acurately produce the amount of animal offspring needed for these different purposes using methods outlined below.

Roughly 200 mice (one fifth over the life of the PPL) are required to test and develop sound methodology. For example, small groups of animals will be needed to establish how to effectively administer medicines, to assess if there is unforeseen harm expected in a planned study, and to test the conditions for tumour growth. These so called pilot assessments are needed in order to accurately plan an ultimate study testing a novel cancer treatment.

Roughly half the mice (500 over the life of this licence) will be needed to ultimately evaluate new cancer treatments and to generate cancer tissue for studies. These numbers assume two studies per year comparing an existing treatment with a novel treatment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Animal numbers will be kept at a minimum through robust experimental design containing appropriate controls and appropriate study size. A clearly formulated hypothesis will underlie each study. Best practice will be applied to run studies, including robust plans for analysis aligned with the nature of the data, the random, unbiased assignment of animals to groups and blinding for data collection, with the aim of avoiding bias, and of producing high-quality trustworthy results.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where possible studies will be run using a fractionated design, where data are collated from independent smaller cohorts. A fractionated study design allows adjustments to flexibly increase study power and frequently improves robustness.

Breeding of animals will be actively managed to avoid surplus pups. This will be achieved by limiting the number of breeding pairs held at any one time as well as by breeding interruptions where the male is removed from its female (s) for some time.

New mouse lines and new crosses that we may generate and that are unavailable through public resources will be made available to the community by archiving them with an open resource.

Mouse lines and their tissues will be shared with other researchers where not precluded by material transfer and legal regulations.

Care will be taken to incorporate all publicly available knowledge relevant to the experimental hypothesis and study design.

Care will be taken that studies do clearly extend, and not replicate existing knowledge.

The outcome of all animal experiments will be published using freely accessible and searchable platforms, regardless of whether the results from the study are positive or negative, with the aim of preventing needless repetition by others.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The overarching aim of the work is to assess novel treatments that are showing promise in cell-based and test-tube experiments in specific situations of cancer where satisfactory solutions are currently not available.

The work under the PPL will exclusively use mice because they are established and well-understood models for the testing of anti-cancer treatments.

The modelling of human cancers in murine backgrounds is highly advanced. This includes the availability of models for specific types of human cancer and established methods of disease monitoring which helps in designing experimental protocols that keep suffering and pain at a minimum.

The behaviour and needs of mice are well researched and understood, including their species-specific needs. Animals will be kept at dedicated facilities in cages that allow them to express normal behaviours. This includes the presence of nesting materials, and the opportunity to hide and forage.

Animals will be kept in natural groups unless scientifically justified.

The mildest possible methods will always be used with cancer models to test the treatments chosen to minimise animal use and prevent them from suffering.

Use of tumour imaging enabelling early stage detection and assessment of internal tumours will permit use of earlier, more refined endpoints

Pilot studies using a reduced, minimal number of animals will be used to test feasibility and methodology. Humane endpoints will be applied, including strict limitations on the amount of cancer that mice may have and pain medicines will be used whenever appropriate.

Why can’t you use animals that are less sentient?

Humans and mice share critical genetic and metabolic features including the ability to develop cancer. Mouse models are a required component in the process of developing treatment protocols for use in patients. Specially bred mouse strains have been generated that uniquely replicate human cancers and therefore are more likely to yield valid and reliable results.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Monitoring: Animals are expected to recovery well from any procedures used under this license. Animals will be continuously monitored until evidence of full recovery, with an absence of signs of pain, distress or ill health. In rare cases where animals may not recover well or exhibit signs of pain, distress or significant ill-health, animals will be humanely killed unless a programme of care can be instituted under veterinarian guidance that leads to full recovery. Animals that have undergone procedures will be assessed daily for a minimum of five days.

Post-operative care and pain management: Pain, suffering or distress associated with procedures will be managed through induction and maintenance of general or local anaesthesia. Sedation and pain relief will be used in accordance with veterinarian advice. All surgical procedures will be carried out aseptically.

Administration of medicines substances or withdrawal of body fluids will be undertaken using volumes, routes, and frequencies that themselves will result in no more than transient discomfort and no lasting harm. The mildest possible strategies will be identified in consultation with the veterinarian surgeon and published guidelines on minimal severity.

Animal handling and care: In all cases, animals will be handled using refined and kind methods, such as using tunnels in which the mice voluntarily enter or cupped hands to remove animals from their cages. Animals will be trained to be handled prior to the start of any procedures. Animals obtained from outside sources will be allowed to grow familiar with their new environment for a minimum of 7 days.

Care will be taken to minimise stress and disturbance coming from the handling of animals. For example, drug administration, weighing and tumour measurement may be combined in a single session to avoid repeat disturbance.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Work under this PPL will follow the Guidelines for the welfare and use of animals in cancer research (Workman et al. 2010; PMID: 2050246), and their planned update once this becomes available.

For transparent reporting of work involving the use of animals, we will use the updated ARRIVE guidelines (Sert et al, 2020; PMID: 32663219).

We will update our work practice with relevant emerging information refinement obtained from attending institutional 3Rs symposia, by attending NC3R workshops relevant to our work programme, and by following relevant advice published by NC3R in their newsletters.

We will follow the guidelines for best practices in colony management collated by the NC3R (https://www.nc3rs.org.uk/3rs-resources/breeding-and-colony-management/colony-management-best- practice).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Advancements to model and study disease situations relevant to the work under this PPL will be actively and regularly assessed through literature searches using relevant keyword searches.

To keep up to date with the latest 3Rs developments I have a subscription to the NC3Rs e-newsletter and regularly search the NC3Rs Webb outlet for updates and refinements opportunities for procedures

205. Functional Analysis of the SRF Network and Rho-Actin Signalling.

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Immunity, Cancer, Transcription, Signalling, Cytoskeleton

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project aims to understand the molecular mechanisms by which physiological, developmental, or environmental signals control the activity of cell's major structural framework, the actin cytoskeleton. The work will elucidate connections between cytoskeletal regulation and cancer development, and in the response to infection. We will focus on the Serum response factor (SRF) network of transcription factors and rho-actin signalling.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Complex chemical signalling mechanisms control cells' physical growth and proliferation, their specialised identity, e.g. as muscle or liver cells, and their interactions with other cells and tissues. The cell's major structural framework, the actin cytoskeleton, is a major target for signals in development and disease. In cancer development, it plays a central role in the process by which cancer cells leave their primary site and establish at remote sites in the body, while in the immune system, it is critical for the generation of immune cells, and for their ability to respond to infectious challenge. These processes are major causes of

mortality and morbidity and understanding them better will in the long- term lead to improved approaches to diagnosis and therapy.

What outputs do you think you will see at the end of this project?

New insights into the principles governing how cells adhere to each other, move through tissues and around the body, and how their growth is controlled.

An improved understanding of:

- the mechanisms of immune cell development, and their functions in the response to infection.

- the mechanisms controlling cancer cell proliferation, invasion, and metastasis.

- the connection between the dynamics of the cytoskeleton and the regulation of gene expression.

These insights will be communicated by scientific publication in the scientific literature

Who or what will benefit from these outputs, and how?

The discovery research community at large will benefit from our increased knowledge of how cytoskeletal regulation impacts on immune and cancer cell behaviour in the animal, increasing our understanding of mammalian physiology and cell biology. Scientists interested in the dynamics of cytoskeletal structure and function may be able to use our new model systems in cell culture for detailed mechanistic analysis.

In the long term, the work may contribute to the development of new approaches to therapy. If our work implicates readily targetable genes or proteins, we will explore its potential for translational application. Our establishment has a philosophy of early and sustained engagement, supported by extensive infrastructure, to maximise translational opportunities arising from its research. Its core-funded Translation Team, has extensive experience in industrial R&D, development, and tech transfer. The team is supported by a Translation Advisory Group comprising experts drawn from clinical medicine, and both small and large commercial sectors.

The translation team manages open-science agreements pharmaceutical companies for early-stage development of its discoveries through researchers based at each partner. A variety of early translational funding streams are available through the various schemes developing ideas into innovation, as well as other Translation Funds. The translation team also advises and provides support about IP protection, and facilitates the development of commercial spin-out companies.

How will you look to maximise the outputs of this work?

For functional studies in mice, we will study our genes of interest in well-characterised infection and cancer models: our findings will thus extend the wider understanding of these models and be relevant to other scientists using them.

Genetically modified animals generated by the project will be made available in advance of publication to other researchers who request them.

Development of improved approaches in the project will be facilitated by sharing results informally at presentations within the Institute, and at national and international scientific conferences.

Results will be publicised formally by publication of peer-reviewed primary research papers following posting on BioRxiv.

Species and numbers of animals expected to be used

- Mice: 25000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Cancer development and spread, and the development and function of the vertebrate immune system, are long-term processes in which different cells interact in a dynamic three-dimensional environment and respond to signals emanating both from their immediate neighbours and distant tissues. It is not currently possible to replicate the complexity of mammalian physiology and tissue structures required for our studies using cultured cell or non-regulated animal models. At the level at which we seek to

understand these processes, mouse models are sufficiently close to the human setting to provide useful mechanistic insight into cancer onset and spread and the development of a functional immune system. Transgenic models will recapitulate mutations seen in human cancers and also allow us to mimic the immune response to infection.

Some aspects of cell behaviour that we are interested in such as basic cell migration can be studied in tissue culture, and genetically modified mice also provide a valuable source of specialised cell types for such studies. Use of "primary" cells – that is cells derived directly from mice that may be cultured in- vitro, for example bone marrow derived macrophages or dendritic cells, is more informative than use of established cell lines, whose behaviour is often altered through their adaptation to growth in culture.
These data inform in-vivo studies where migration can be assessed in real tissue environments, for example, how cells migrate from the vasculature into and within organs. This is important for our fundamental understanding of cancer and immune biology.

We will generally use young adult mice for our studies, although for cancer development studies mice will be kept for longer periods.

Typically, what will be done to an animal used in your project?

The majority of mice will be used for breeding to generate specific combinations of genetic mutations, the majority of which will not produce harmful effects (95%). A minority will develop spontaneous tumours which may have adverse harmful effects or be predisposed to the development of tumours upon administration of gene altering substances, again which may have adverse harmful effects.
Offspring bearing relevant genetic modifications will be culled and used for tissue analysis or the generation of cultured cells for functional analysis.

For cancer studies, mice of particular genotypes will be injected with cancer cells to study tumour development or colonisation of remote sites. Some genetic combinations will render the animal liable to develop cancers, such as melanoma or mammary tumours. In these models, cancer development will be either spontaneous, or conditional on administration of an inducing substance. Surgical procedures, for example the removal of primary tumours, may also be performed on a subset of mice.

Immune cell development and migration will be studied in mice carrying specific genetic modifications. To allow study of genetically modified immune cells alone, some mice may undergo immune reconstitution following irradiation of animals or be injected with genetically modified immune cell populations. Studies of the immune response to infection will involve well-characterised infection models, such as Listeria monocytogenes. Extensive post-mortem tissue analysis will be performed to maximise the information obtained from each animal.

The duration of experiments is very dependent on the investigation in question. For example, infection studies will typically last from several days up to two weeks, longer if an immune rechallenge experiment is required. In contrast cancer onset and development studies are typically longer. In melanoma studies these may be from 12 weeks up to 12 months. The duration of experiments will be kept to a minimum to minimise harm while meeting the scientific objective.

For each Protocol the number of procedures will be kept to a minimum to achieve the scientific goal. Mice will be culled by Schedule 1 killing at the end of experimental procedures.

What are the expected impacts and/or adverse effects for the animals during your project?

Most mice used in breeding protocols will not experience any adverse effects, other than mild transitory effects, for example tissue sampling to assess genotypes or biopsy, and will not be subjected to further experimentation.

Some animals will experience adverse effects through tumour development, arising as a result of inherent genetic predisposition, cell transfer, or carcinogenesis. Any animal displaying adverse will culled within 24 hours. The severity in these experiments, which will be conducted in accordance with the National Cancer Research Institute guidelines, is not expected to exceed moderate. Tumour size will be carefully monitored and not exceed 1.2cm diameter. In experimental metastasis models, animals will be culled within a defined time period (depending on the cells under study) and this is expected to be prior to the onset of adverse effects. However, animals will be culled sooner if they show signs of ill health.

Some animals will be subject to more complex procedures, such as bone marrow reconstitution. This procedure is tolerated well, some mice may exhibit a general malaise but are expected to recover quickly. With Infection regimes and doses of pathogen will be chosen so as to minimise adverse effects. For example, in our experience intravenous infection with low levels of Listeria monocytogenes does not result in adverse effects, with the infection being cleared by day 3.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Animals will experience only mild severity, under the breeding and maintenance protocol. Moderate severity may be experienced in a minority of spontaneous tumour models, induced tumour models and some immune studies. In almost all cases, scientific data will be collected before moderate severity is encountered. Although difficult to estimate, this would not be expected to exceed 10%. Moderate severity may also be assigned due to cumulative steps within protocols, again this is not expected to exceed 10%. For some methods of tissue sampling or processing, animals may be culled under terminal general anaesthesia.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cancer development and spread, and the development and function of the vertebrate immune system, are long-term processes in which different cells interact in a dynamic three-dimensional environment and respond to signals emanating both from their immediate neighbours and distant tissues. Non- protected organisms, or simple tissue culture models cannot recapitulate such complex events, and the use of animals for some studies is therefore essential.

Where possible we will use "primary" cells derived from mice and cultured in vitro to gain mechanistic insights of cell behaviour. In some instances we use human cell lines in metastasis studies. However, at the moment the complexity of our immune genetic models (multiple gene modifications) cannot be recapitulated in human primary cells.

Which non-animal alternatives did you consider for use in this project?

Many of the aspects of cell behaviour that we are interested in will be studied in tissue culture systems using cells lines, for example, NIH/3T3 cells and mouse embryonic fibroblasts and we will continue to use this approach. We have considerable expertise in such assays and will use them in place of animals wherever possible. We are also working with mouse tissue spheroids and organoids. This may provide an in vitro approach to for example, to investigate how skin tumour cells interact with each other. However, it is essential to also study primary cells generated from genetically modified animals that display defined characteristics, such as macrophages, T-cells and dendritic cells. Recent technical advances allow specific "editing" of genes in cell cultures from normal mice, and where feasible we will apply them to our culture models, reducing the need to generate genetically manipulated mice.

However, this is not always possible making conventional breeding essential for this project.

Why were they not suitable?

It is not currently possible to replicate the complexity of mammalian physiology and tissue structures required our studies using in-vitro culture models or non-regulated animal models. Thus, the study of cancer development and spread requires a system in which cancer develops within a tissue and becomes disseminated to other organs, in a setting that is close to that seen in humans. Similarly, the immune system can only be functionally analysed in an organismal context: cells migrate between and within tissues during development, and immune cell function within the organism is dependent on dynamic migration between tissues and lymph nodes via the vasculature. Simpler non-regulated animal models are thus not appropriate to realise our objectives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This estimate is based on our experience of previous usage under our current PPL. Estimates are based on the required level of breeding to generate defined genotypes. We do, wherever possible breed multiple conditional homozygous alleles to minimise the number of animals bred for our experiments. In addition, estimates are based on our previous experience of technical variation with our various analysis methods, for example characterisation of immune cell analysis by flow cytometry, or quantitation of tumour growth and metastatic spread, and the expected magnitude of the anticipated effect. Most experiments involve 3–6 mice per genotype, three independent determinations, and consist of a control and experimental groups, with occasional simultaneous analysis of multiple groups. If prior in vivo data is not available, experimental design will be informed by combination of cultured cell data generated in the laboratory, appropriate literature, and our experience.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Use of new genome editing technologies to generate mouse strains will reduce the amount of breeding required. We will also use these techniques to modify genes directly in primary cells cultured from WT animals, increasing the scope of our culture experiments, and ensuring that only the most informative mutations are tested in animal experiments. Breeding pairs will be designed to maximise the generation of useful offspring within the confines of biological limitations (for example infertility)

Wherever possible we will use the mice that we breed to produce multiple data outputs, and/or cells for culture studies. Immune reconstitution and adoptive transfer approaches will aim to maximise the information obtained from complex genetic crosses, reducing breeding. In our cancer studies we will minimise animal numbers used by tracking tumour development using imaging approaches.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Mouse lines are routinely maintained by keeping 2-3 breeding trios. To minimise breeding, lines under sporadic use are maintained at lower levels, and frozen whenever practicable. Lines will be maintained in collaboration with other licences wherever possible to minimise breeding.

Around 40 strains are maintained. Strains will be maintained at a minimum viable level, and this continually monitored. Where lines of investigation are paused to accommodate emerging priorities, embryos are frozen and live lines removed. The number of strains reflects the complexity of the genetic combinations underlying the immune and cancer models. For example, the melanoma studies may require the maintenance of 8 breeding strains to generate 4 experimental strains bearing spontaneous tumours or inducible tumours, alongside appropriate controls. This is to ensure harmful combinations of genetic mutations are only created for specific studies.

Where possible, mice will be bred to homozygosity to enable all offspring to be experimentally relevant. In some models, however, only 50% or 25% of offspring are useful (e.g., infertility of homozygous females, or inability to nurture young).

Use of new genome editing technologies to generate mouse strains will reduce the amount of breeding required. We will also use these techniques to modify genes directly in cells cultured from normal animals, increasing the scope of our culture experiments and ensuring that only the most informative mutations are tested in animal experiments.

Mice will be made available to other researchers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are most appropriate for our studies because of the ease with which they can be altered genetically. Their biology is close enough to that of humans to allow the generation of mouse infection and cancer models that closely reflect either specific human disease states or specific cellular interactions that occur in the human setting. Examples of tumour models include the use of mice prone to mammary tumours, melanoma, or lung cancer. These are the most refined models available to study the mechanisms of cancer onset and spread allowing valuable new insights into the role of the SRF pathway, RPEL protein in these processes whilst causing the least amount of harm by employing defined timelines of tumour progression and humune endpoints. For immune studies, we use infection with

listeria to track the cellular immune response in vivo. Listeria is tolerated well and cleared by mice in the doses that we will be using.

The basic models that we will employ have been extensively studied by others investigating different genetic pathways. This will allow us to build on these models and apply our investigative approaches with specific reference to the role of SRF and the RPEL proteins in cancer and immunity.

Why can’t you use animals that are less sentient?

It is not currently possible to replicate the complexity of mammalian physiology and tissue structures that are required for our studies using animals that are less sentient. Overall, we need to use young adult mice and older for cancer studies and immune studies that require a fully developed and responsive immune system. Less sentient models do not replicate the structures and systems in our cell migration, cancer and immune studies, and are thus not appropriate to realise our objectives.
Although zebrafish are proving increasingly useful in immune studies, some of the specific processes are do not faithfully recapitulated in zebrafish models.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We always try to minimise any possible adverse effects. Regular monitoring by our animal care staff will be tailored to the specific type of model under study, for example superficial tumour studies will

involve regular monitoring of tumour size so as not to exceed 1.2cm diameter; internal tumour models including experimental metastasis and lung tumours will be carefully monitored for the general well- being of the animal to include dyspnoea, weight loss and any deviations from normal behaviour.
Experiments will be designed to run for the minimum length of time to generate valuable scientific data with the earliest endpoints possible. Each step of each Protocol will incorporate well defined humane end points. Additionally, we strive to keep updated with the latest environmental improvements, such as enhanced environmental stimulation.

All the work will be performed by highly trained personnel and undertaken under conditions in which animal welfare is a priority. Models for both cancer and immune studies and interventions do not exceed moderate with valid scientific endpoints are reached before humane endpoints in almost all cases. Animals will be monitored frequently for signs of distress and appropriate action taken promptly.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We keep abreast of developments in the NCRI Guidelines for the welfare and use of animals in cancer research, and LASA guiding principles for aseptic surgery. We discuss potential refinements with colleagues in other research groups, our NACWOs, and veterinary staff. We remain up to date with the latest published literature allowing us to employ the most refined protocol to deliver the scientific objectives.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay up to date via regular communication with animal house staff, other scientists in the field, consultation with our NC3Rs programme manager, and regular visits to the following website https://www.nc3rs.org.uk/3rs-resources and the ARRIVE guidelines. We also receive regular updates from our internal communications.

206. Gene Regulation in White Blood Cells in Inflammation of Blood Vessels, Heart and Lungs Leading to Heart Attacks

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
- Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

macrophage, iron, gene, regulation, inflammation

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We are interested in developing drugs that will prevent heart attacks and strokes, which are leading causes of death and disease in the world. It is now understood that inflammation - like the response to an infection - mediates the disease of vessels that leads eventually to heart attack and stroke. The drugs and understanding that we generate will also be useful to treat inflammation, which plays a role in many other diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Heart attacks and stroke are a major cause of death, suffering and disability in humans. These are most frequently due to fatty deposits in the walls of blood vessels supplying the heart and brains. The fatty deposits cause inflammation and hardening, but also weakening leading to the wall breaking down and triggering a blood clot that blocks blood flow. A key event is that a slight degree of breakdown of the vessel wall allows blood to enter the vessel wall. As blood pigment is toxic to tissues, this makes the inflammation rapidly worse leading to much worse breakdown, blocking blood flow. This work is aimed at improved diagnosis and treatment, concentrating on ways to both reduce inflammation and reduce toxicity of blood pigments.

Some strokes comprise large bleeds within the brain. As we are studying mechanisms of clearance of tissue bleeds, our work will also help with those strokes that are not due to fatty deposits in the arteries leading to the brain.

We concentrate on one particular aspect, which is the regulation of a molecular machine that deals with the blood pigment that carries oxygen. Blood pigment is toxic to tissues if released from red blood cells. This means that it is important to rapidly increase the levels of the molecular machinery to degrade and recycle it in the event of release. Indeed, blood pigment released into the walls of blood vessels is likely to trigger worse inflammation, triggering a heart attack or stroke. This happens because some of the blood that the vessels are transporting can leak into its wall if the wall becomes weakened and leaky from carrying too much fat.

We think that activating this safety system using molecular levers to switch on the disposal machinery may allow us to prevent heart attacks and strokes.

One of the Aims is additionally to understand the impact of air pollution on heart attacks and strokes, and how air pollution works in that respect. This will allow mitigation of the effects of air pollution.

Some of the work we plan will allow improved understanding of the body's natural command and control systems for this molecular machine. Other experiments will be necessary to show initial proof of concept that new drugs may prevent heart attacks by activating these command and control systems. However, it is likely that the final versions of these drugs will be slightly different, so we are not planning to engage in experiments directly required for drug licenses. When we come to that aspect, we would work via specialist contractors.

What outputs do you think you will see at the end of this project?

1. Large-scale human genetic studies have pointed the finger at a number of genes in heart attack and stroke. This is largely on the basis of their location. These studies will show whether or not they directly cause heart attack and stroke and, importantly, how.

2. We will validate new potential cardiovascular drug treatments based on refinement of existing anti- cancer drugs. These work by activating molecular levers on genes that switch them on and off.

3. We will show the utility of a new cardiovascular diagnostic based on the light emission from a natural molecular machine (enzyme) for blood pigment. This molecular machine removes blood pigment from body tissues.

4. We will study whether licensed drugs used in high blood pressure in the lungs, and skin inflammation, may be repurposed to prevent heart attacks and strokes.

5. We will have defined a new mechanism of gene activation. In this, metabolic signals directly alter the proteins wrapping DNA, and alter the targetting of tiny switches that activate genes (called transcription factors).

Who or what will benefit from these outputs, and how?

Humans will benefit from the diagnostic reagent being developed on a timescale of years and from the therapeutic reagents being developed on a timescale of a decade. The knowledge gained from the experiments may permit repurposing of existing licensed drugs, which may allow more rapid benefits to human health.

Other researchers in the field will benefit from the published knowledge.

How will you look to maximise the outputs of this work?

We will publish the work in prestigious journals and involve the press offices of the funders and institution in issuing press releases.

Species and numbers of animals expected to be used

- Mice: 2000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the simplest model we could select that would be representative of the complex processes. This is because we study heart attacks and strokes. At its simplest, only a complete organism will get a heart attack or a stroke. Mice and humans are similar and mouse inflammatory and vascular disease resembles human inflammatory and vascular disease.

Adults are chosen as heart attacks and strokes affect adults.

Typically, what will be done to an animal used in your project?

Atherosclerosis. Mice will be bred to develop a propensity to heart attack and stroke, but experiments will be stopped at a point early enough to prevent any suffering. Use of sophisticated measurements will still allow analysis of preventive treatments.

Air pollution will be studied by briefly putting the animal to sleep. This is done to get the animal to cooperate. Air pollution will be added to the airways and the animal wakes up after a few minutes. After a few days to weeks, the animal is euthanized and its effects on blood vessels measured, including an analysis of how that effect is brought about.

Removal of blood and inflammatory cells from tissues will start with briefly putting the animal to sleep to get the animal to cooperate and to help make the procedure accurate. Then an injection under the skin, similar to daily insulin injection of a diabetic is carried out. Then after a few second to minutes, the animal wakes up, is kept for a few days and is then euthanised and tissues analysed.

Effects on inflammation will be studied using a more refined version of a standard model. In this, the animal will be briefly put to sleep, and air injected under the skin. This will be followed up with an injection of a small amount of a purified inflammatory agent, not enough to trigger unpleasant whole- body effects (e.g. fever). Then the animal will be kept for up to about a week and then euthanized and tissues studied.

In some experiments, the most humane and quickest approach to some questions will involve replacing white blood cells. This will be done in about 20% of experiments. In this the animal is exposed to radiation, which is itself painless. The procedure is made safe by antibiotics whilst new white cells are regenerating, and very promptly injecting new white blood cells from another animal (eg missing the gene under study). This involves only an injection. the principal risk, which is infection, has been minimised by careful optimisation of the procedure.

About 10% of experiments will involve surgical implantation of a drug-releasing implant immediately underneath the skin. This will be done by briefly putting the animal to sleep, making a tiny cut and inserting the implant. The animals will be allowed to wake up again and recover. There will be plenty of analgesia given before and after the procedure. The procedure will take a few minutes to tens of minutes and will be done once. The implant will stay in place for up to 6 weeks.

Imaging will start with briefly putting the animal to sleep, photographing and then allowing to wake up again. This will take a few minutes to tens of minutes. It may be done once each day on 4 occasions. This will be done in about 10% of experiments.

What are the expected impacts and/or adverse effects for the animals during your project?

With one of the genetic models, neurological symptoms, sudden death and heart failure is seen in older mice on high fat diet but the mice will be culled before that point.

The main problem with the replacement of white blood cells is infection during the replacement period. The period of sensitivity is for about a week and is covered with antibiotics and close monitoring.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Based on severity estimates in the downstream experimental protocols, approximately 600 are expected to be moderate out of total of approximately 2000 (approximately 30%).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We have already built up as much data as we can with human cells kept alive in plastic dishes. This has given us several good new ideas how to target processes leading to heart attacks and strokes. Heart attacks and strokes are the product of complex interactions between multiple body systems.
Therefore, we need to show these ideas work in a complete organism.

Which non-animal alternatives did you consider for use in this project?

We routinely work with human cells kept alive in plastic dishes, and target genes using modern sophisticated approaches involving genetic modification and gene-based measurement. At this stage, we have done as much as we can with this approach. We have successfully identified strong hypotheses and potential drug approaches to prevent heart attacks and strokes.

The next step needs to capture complex interactions between multiple body systems such as vessels, the immune system and metabolism and reproduce human heart attack and stroke as accurately as possible without causing significant actual harm.

So-called 'organ-on-chip' and 'organ slice' approaches were considered but are still in a prototype stage for even short-lived assemblies for anatomical validation and do not capture disease, inflammatory disease, or long term effects.

Why were they not suitable?

They were suitable for getting ideas about how the diseases and drugs work. But to prove the ideas work in diseases of complete organisms, such as stroke and heart attack, will require complete organisms.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We calculate that a typical experiment to show an interesting effect of a drug, and its mechanism, would require 32 mice, carrying out the work steadily over 5 years. We have based these numbers on statistical estimates of how strong our scientific conclusions can be, given the known levels of variation between mice .and the estimated effect sizes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used the NC3Rs Experimental Design Assistant and statistical advisory packages, and statistical advice from leading journals.

One particular point is that we have recently been advised in the use of a repeated measures ANOVA by Circ.Res. statistical reviewers. This incorporates multiple measures on an individual (particularly in vitro) and builds in individual identity to make the statistics more powerful and the n-value less.

The work will have a substantial in vitro development phase, which allows us to conduct the absolute minimum number of experimental interventions in vivo due to targetting highly probable candidates in the first instance.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use efficient breeding, computer modelling, pilot studies and sharing of tissue.

This allows us to conduct the absolute minimum number of experimental interventions in vivo - as well as refining those interventions (mainly) to the point of being subclinical. This is due to targetting highly probable candidates in the first instance.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Only a complete organism will get a heart attack or a stroke. Heart attacks and strokes do not meaningfully occur to non-mammals. Mice are the simplest model we could select that would be representative of the complex processes. Mice also breed easily with least harm from breeding of genetic alterations. The models have been refined to a minimal level that is well tolerated.

Animals will be humanely killed shortly before symptoms or severe disease occur. The disease will be measured using sophisticated methods that allow assessment of cells and tissue rather than symptoms. Potentially unpleasant procedures will be carried out under anaesthesia and with plenty of analgesia.

Mice and humans are very similar. Rodents and primates are more closely genetically related than other mammals. Their immune systems, inflammation and metabolism are closely related. For example, human hereditary metabolic diseases cause very close parallels in mice. Although mouse vascular systems are different to humans this is mainly due to smaller size. Mice breed easily and quickly and may be easily genetically modified.

Adults are chosen as heart attacks and strokes affect adults.

Why can't you use animals that are less sentient?

Heart attacks and strokes do not occur in non-mammals or in embryos, and take a long time to develop.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The mice at highest risk will be monitored daily for symptoms and then culled if any symptoms are identified. Any surgery will be minor and whilst asleep with anaesthetic gas with plenty of analgesia afterwards.

We already carry out much of our work in non-animal alternatives including human blood-derived inflammatory cells and human tissue samples of diseased arteries. This is taken to a depth of analysis that is cutting-edge. There is substantial associated computational analysis that extends to sophisticated computer modelling. Our computer modelling has already shown benefits in 3Rs by accurately focussing analysis on one particular gene reducing the number of different knockouts and drugs to investigate.

Mice will routinely monitored by inspection weekly as a minimum and monitoring stepped up if there is reason to suspect increased severity.

We are focussed on pre-symptomatic measurements to assess the preventive interventions reducing severity.

Pre-emptive culling will be used to refine severity especially applicable in Protocol 6, but also applying to other protocols.

Protocols 7 and 8 focus on subcutaneous injections. Moreover, this procedure is carried out under anaesthesia. This approach has refined the severity. The substances injected (cells or labelled biomolecules to be cleared; drugs or genetic modifiers) will be refined with respect to dose, volume, side-effects, specificity, all of which are refining aspects.

Each protocol has provision for replacement of the bone marrow and white blood cells with genetically- modified matching white blood cells. This has been rendered free of significant harm by splitting the radiation dose, using the minimum radiation dose, prompt injection with rescue white blood cell precursors at significant number, and covering with acidified sterile drinking water containing antibiotics, and sterile bedding and pathogen-free cages.

The majority of work under the protocol will examine novel drugs that amplify the putative protective mechanisms and themselves constitute refining agents. The work will have a substantial in vitro development phase, which allows us to refine those interventions (mainly) to the point of being subclinical. This is due to targetting highly probable candidates in the first instance.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the NC3Rs PREPARE and ARRIVE guidelines, and NC3Rs resources more generally. We will also follow LASA and College guidelines. Where applicable, we will follow any specific directions by NVS.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

CPD via 3Rs programme. Continuous feedback and thought about refinement approaches. Searching and reading of the literature (eg PubMed) regarding approaches that might help with 3Rs.

Work closely with NTCO to ensure that skills of all carrying out procedures are as up to date and refined as reasonably achievable. This would apply to myself and all all team members working with specified procedures.

207. Production, Analysis and Maintenance of Genetically Altered Livestock

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Transgenic, Genome editing, Disease resistance, Disease model

Animal types	Life stages
Sheep	embryo, adult, neonate, juvenile, pregnant
Pigs	embryo, adult, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The ability to manipulate the livestock genome – be it genetic modification involving insertion of a transgene or precise manipulation of endogenous genes with genome editors – is a powerful tool for both the progression of basic scientific research and applications to advance biomedical and agricultural industries. This programme of work enables the production, preliminary characterisation and where appropriate the maintenance of several different livestock models that fall into two broad areas: (1) reliable models of human disease, and (2) genetically altered livestock relevant to agriculture. This PPL progresses maintenance and analysis of animals made during our previous service license, and where appropriate allows new models to be created (for which amendments to this license will be sought).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The aims of this project relate to the creation of specific changes to the genes of pigs or sheep. They include experiments to make livestock that are resistant to diseases that blight the industry, the creation and analysis of models of human diseases (where rodent models are currently insufficient), and refinements to the way we make genetic changes to animals.

Diseases of livestock are a huge economic burden on the farming sector, and can result in considerable uncertainty in production systems. Our work on disease resistant animals should benefit agriculture (both farmers and animals). Discoveries made under our last project license have been licensed by an international animal breeding company, and are progressing through regulatory submission in the USA. We will continue to characterise animals made under our previous license and will seek to assess new models (with amendment to this license) as new targets are discovered.

Given increased global temperatures associated with global warming, improved thermal tolerance in livestock is a desirable goal. Mutations within the prolactin receptor gene of cattle are associated with improved thermal tolerance in conditions of elevated temperature and humidity. We will now seek to introduce similar changes into the genome of sheep and assess the consequence of altered gene expression on wool growth and thermal tolerance.

Animals are often used by scientists as models of human diseases, allowing us to better understand disease processes or to test new treatments. Mice are commonly used for this purpose, but either the biology of mice or their small size can sometimes make them inadequate models. Our livestock models of human diseases aim to bridge this translational gap, allow better understanding of disease states and progression and providing a platform for pharmaceutical companies or charitable organisations to better evaluate therapies that they have developed in other systems.

Ongoing examples include:

Batten disease is a group of closely related genetic disorders that result in the death of cells in the brain resulting in death of affected patients. Mutation in a gene called PPT1 causes the most severe form of this disease, with affected children dying before puberty. Mouse models increased our understanding of this disease, but differences in brain size and structure meant it was difficult to accurately model the human disease state in rodents. We created a sheep model of Batten disease, as sheep have a brain which is not only larger than that of a rodent, but also structure which is more similar to humans. We initiated characterisation of this model under the previous license, and supplied animals to an additional license for translational studies. We seek to progress this work under this license.

CDKL5 Deficiency Disorder (CDD) is a relatively common (1:41,000) genetic disease associated with early-life epilepsy. While rodents can be used to model some aspects of CDD they do not develop epileptic seizures. In order to understand whether epileptic

seizures are limited to the human condition or are associated more directly with brain size we edited the CDKL5 gene in pigs, which have a much larger brain than rodents. We are characterising these pigs to assess whether they develop a disease similar to that of humans. If successful this may lead to translational studies (subject to license amendment).

Microvascular diseases such as vascular dementia are a major cause of morbidity in aged humans, with few treatment options. While rodents such as mice and rats have been useful in studying these diseases, livestock models such as pigs and sheep are likely to offer more accurate models as their cardiovascular, immune and central nervous systems are all more similar to humans. We have made transgenic pigs in which we can induce high blood pressure in order to better understand these diseases and provide a model system of appropriate size and complexity to allow novel therapeutic approaches to be trialled. These pigs will be maintained on this license and supplied to another license for induction studies.

SARS-CoV-2, the virus that causes Covid-19, enters human cells by binding to a protein on the cell surface called ACE2. While the spike protein of SARS-CoV-2 binds to human ACE2 well, it does not bind so well to the ACE2 protein of mice or pigs. As a consequence it is difficult to infect these species with SARS-CoV-2. Transgenic mice expressing the human ACE2 protein have been made elsewhere, and have proven valuable in studying the disease. As mentioned previously, the size and anatomy of pigs makes them a superior model of human diseases than comparable rodent models. We have made transgenic pigs expressing the human ACE2 protein, and anticipate that this will be a superior model to aid our understanding of this pandemic disease, as well as model of suitable size to allow investigative intervention studies with translational potential. Transgenic pigs will be maintained on this license and supplied to another license for disease studies.

What outputs do you think you will see at the end of this project?

We have a track record for the discovery of useful intellectual property. Under our last project license we discovered a novel genetic alteration that made pigs completely resistant to one of the most

important diseases of the pig industry. This discovery has been licensed by an international animal breeding company, and is under regulatory scrutiny for market entry. We are progressing similar projects under this license, and anticipate the discovery of new genetic alterations that will benefit the livestock breeding sector. In addition to intellectual property this will also result in research publications.

Our livestock models of human disease bridge a gap between data generated in rodent models and human disease states. By characterisation of disease progression in our models we can better understand disease progression in humans. In addition, the relative size and complexity of the livestock models allows translational intervention (under separate license authority) with therapies developed elsewhere. Much of the data generated will be novel and thus suitable for scientific publication.

Who or what will benefit from these outputs, and how?

Our work on disease resistant animals should benefit agriculture (both farmers and animals). We are progressing projects on disease resistance and environmental resilience

that are relevant to national and international farming. Several edited livestock products have already been approved for commercial sale (North America, South America and East Asia). With ongoing international developments in the regulatory frameworks governing genome editing in livestock (including in the UK) we anticipate such approaches will become increasingly common as part of commercial livestock husbandry practices within the next 5 years.

Our models of human diseases aim to allow pharmaceutical companies or charitable organisations to better evaluate therapies that they have developed in other systems. Several of these models are already under commercial evaluation under separate license authority, while others are at an earlier stage of development. All of the proposed models of human disease have the potential to overcome limitations of existing animal models and provide a tool to both evaluate therapies and further improve our understanding of these diseases.

It is anticipated that all models evaluated on this license will generate novel scientific data that will benefit the research community.

How will you look to maximise the outputs of this work?

Many of our projects are multidisciplinary, involving scientific colleagues from both other academic institutions and industry.

The institute primarily involved in this research has world class facilities and a track record of delivering cutting-edge research. It is the UK's foremost centre for livestock genetics and genomics and a world leader in methodologies for analysing and manipulating animal and pathogen genomes. The Institute excels in both basic and translational research that tackles pressing issues in animal and human health and welfare. These projects will make use of an animal research and imaging facility which is fully supported and maintained by dedicated animal services and expert technical personnel.

Our academic collaborators are based both within UK research institutions and internationally. Our industrial partners all have a global footprint. Dissemination of new knowledge will be within this

framework, as well as more broadly within the scientific community. Due to the broad reach of the collaborative frameworks there is a greater chance that discoveries will result in real-world products.

Species and numbers of animals expected to be used

- Sheep: 350
- Pigs: 160

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All animal experimentation raises ethical concerns. We will always use the most appropriate species/sex/age of animal to answer the question being posed. Some of the projects on this license address species-specific questions (eg. will modification of a specific molecular chaperone impact progression of a viral disease). For other projects we are asking questions about comparative physiology. In these circumstances, where existing rodent models are insufficient to adequately answer the questions posed, the more appropriate scale and structural similarities between sheep/pigs and humans offers opportunities to perform more translational science.

Choice of species for any project on this license is discussed in advance within our group, with collaborators, with our NVS and NACWO, with our AWERB and with the Home Office Inspector(s). These discussions result in refinement in the specific scientific aim of the genetic alteration and the methods of data gathering. Most importantly, this discussion serves to minimise the potential for adverse effects associated with the project.

Typically, what will be done to an animal used in your project?

There are a number of different workflows on this license, so there is no one typical experience that an animal will undergo.

Some animals will be used as embryo donors. For this they will be injected with hormones in order to stimulate them to come into oestrus. Pigs will be artificially inseminated then culled, and embryos collected for genetic alteration. Sheep will be culled and eggs collected, and these will then be matured and fertilised in the lab before genetic alteration.

Some animals will be used as embryo recipients. For this they will be injected with hormones in order to synchronise their oestrus cycle with donor animals. They will then be anaesthetised and a small incision made in their abdomen to expose their oviducts. Genetically altered embryos will be transferred into the oviducts and the opening sutured closed. Good surgical and aseptic technique, along with appropriate pain relief and antibiotics, means that any discomfort is moderate and transient. If animals fail to become pregnant they may be used as embryo donors or they may be culled. If animals become pregnant then they will be maintained until their offspring are weened, at which point they will be culled.

Some animals will be used for breeding purposes, either to maintain the genetically altered population for studies on this license or to supply them to other research licenses. In this case they will not usually have any procedures performed on them. On rare occasions a blood sample or tissue biopsy (with appropriate anaesthesia and pain relief) may be taken in order to confirm the nature of the genetic alteration (eg to confirm a transgene is expressing). In such instances appropriate anaesthesia and pain relief will be provided to minimise stress and pain, and any discomfort will be moderate and transient.

Some animals will be used for experimental purposes on this license. This may involve the collection of blood and / or tissue biopsies on up to two occasions, or scans such as MRI or CT with an upper limit of 10 cycles (with repeat imaging only performed once the animals has fully recovered from the last round of imaging). Appropriate anaesthesia and pain relief will be provided in association with each of these procedures in order to minimise pain and distress to the animals.

Most animals on this license will not develop disease as a direct consequence of their genetic alteration, but some will. When creating models of human disease it is anticipated

that a human-like disease state will develop. One of our projects is investigating a neurological disease which results in epilepsy in children. The same genetic variation does not cause epilepsy in rodents. We are seeking to understand why by introducing similar genetic variation into pigs, and as a result our pigs may develop seizures.

What are the expected impacts and/or adverse effects for the animals during your project?

There will be minimal adverse effect to animals used as embryo donors, as they will be injected with hormones and then culled prior to collection of eggs / embryos.

Animals used as embryo recipients will be injected with hormones, anaesthetised and undergo minor surgery. This will have an adverse effect, which will be minimised by the application of appropriate anaesthesia and pain relief.

Pigs and sheep are housed indoors on our research farm all year for biosecurity. The majority of animals used in this project will be involved only in breeding, with no ill effect anticipated as a result of either the procedure or their genetic alterations. Some of the animals involved in human disease modelling are likely to become sick. For new models where we do not know how fast disease progression will occur, we will monitor animals closely throughout life, including with remote camera recording where a significant risk of disease development is anticipated. Once the first few animals (3-5) reach a pre-defined point (e.g. the first sign of significant disease) they will be killed and samples taken for analysis. Subsequent animals will be killed before they reach this point unless there is specific scientific need.

Sheep and pigs will be anaesthetised using refined protocols discussed with the NVS, NACWO and Veterinary Anaesthetists. Both species cope well with anaesthesia.

Bronchoalveolar lavage will be used to take samples from the lung. Animals will be anaesthetised throughout and it is our experience that they experience no apparent adverse effects from this procedure.

For MRI and PET-CT imaging (to look at the brain or other internal organs) animals will be anaesthetised and remain unconscious throughout the scanning process.

Collection of blood will adhere to our institue's guidelines on volumes and frequencies and will not cause effects that are more than mild and transient.

Collection of tissue biopsies (eg skin) can cause pain, which will be controlled by the application of appropriate anaesthesia and pain relief.

One risk that all of the above surgical procedures have in common is infection. However, our good aseptic practice means that this is very rare. At the end of these procedures some animals will be retained for breeding and some may be moved to other projects, but most will be killed at the end of their use, for example to provide tissues for analysis.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Animals used as embryo donors will experience mild severity. This will impact approximately 10% of animals in this programme of works.

Animals used for as recipients for embryo transfer will experience moderate severity. This will impact approximately 5% of animals in this programme of works.

Animals from which we take a tissue biopsy (skin or lung) will experience moderate severity. This will impact approximately 10% of animals in this programme of works.

Animals from which we will take blood samples for analysis will experience mild severity. This will impact approximately 5% of animals in this programme of works.

Animals that develop an adverse phenotype as a result of a genetic modification are anticipated for some projects, but their numbers will be kept to the minimum required for the scientific outputs (5%).

Animals on which we will perform no invasive procedures (eg they are used only for breeding, or are transferred to another license) will constitute the majority (70%) of animals associated with this license, and their lifetime experience on this license will be subthreshold.

Some animals will experience more than one procedure (eg blood sample and tissue biopsy), hence the numbers above to not total 100%.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Where possible we do use alternatives to animals. For example, we can use computer analysis of the large amounts of genetic information that is now available to identify which genes are likely to be involved in disease processes in both humans and in farm animals. Our initial experiments usually involve cells, where we refine methods of genetic manipulation and ask basic scientific questions, and we also base much of our research upon published preliminary work from other research groups.

For projects aimed at manipulating livestock genomes for potential agricultural application, production of genetically altered livestock is the final step in a long process. Animals are more than a collection of cells, so to fully understand the impact of a genetic alteration on an animals' interaction with its environment there is no alternative to using whole animals.

For projects aimed at better understanding and treating human diseases we only ever create new animal models where a specific need exists. In these instances much earlier

work has usually been done in other systems (eg cells or rodents), but gaps in our knowledge remain either because these models do not always accurately replicate the human condition, or because differences in size and physiology prevent testing of novel therapeutic approaches.

Which non-animal alternatives did you consider for use in this project?

For the studies involving human disease states, much work has already been performed in cultured cells, invertebrates (where appropriate) and rodent models.

For studies addressing questions associated with viral diseases of livestock, exhaustive work has been performed in cells in culture.

When asking how a genetic alteration will impact on specific interactions between an animal and the environment there is often no alternative to using a whole animal.

Why were they not suitable?

We only make genetically altered animals where current alternatives are inadequate to answer the questions posed. We have a number of projects that model human disease states. For each of these much information has been gained through study of cultured cells or rodent models, but in each instance we have reached a position whereby the current rodent models either fail to adequately reflect the human disease condition due to divergent physiology, or the size of rodents proves to be a barrier to therapeutic development.

For studies of livestock disease there is much that can, and has been learned by studying the disease in cells in culture. However, animals are significantly more complex than cultured cells, so in order to understand the impact of a genetic alteration on a whole organism there is no alternative to studying a whole genetically altered animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For each of our projects, approval by the local Animal Welfare and Ethical Review Committee is only given where the animal numbers have been demonstrated to be the minimum consistent with deriving appropriate scientific data.

Our ongoing work with a sheep model of Batten disease is producing very promising data (on another license), involving research teams both within our institute and within industry. We estimate that to provide sufficient sheep for these translational studies over a 5 year period we will use approximately 250 sheep over this period.

Our work involving thermal tolerance in sheep is new and will require 2 generations of animals over a 4 year project. We currently anticipate that we will use approximately 80

sheep over this period, including approximately 30 pregnant females (transferred from our previous license), 30 lambs (first generation) from which we will choose animals with appropriate genetic alterations to breed 20 lambs (second generation) for study.

Our work involving inducible hypertension in pigs will require approximately 20 animals over 2 years. Sixteen will be supplied to another license for studies involving induction of hypertension and subsequent assessment of the impact this has on neural pathology, with 4 transgenic males maintained on this license for subsequent breeding purposes. If we choose to propagate these animals (eg if initial studies show significant promise) we will submit a license amendment which will inevitably increase these numbers.

Our work involving a model of SARS-CoV-2 will require approximately 10 animals over 1 year. Six of these will be supplied to another license for a disease challenge experiment, with 4 transgenic males maintained on this license for subsequent breeding purposes. If we choose to propagate these animals (eg if initial studies show significant promise) we will submit a license amendment which will inevitably increase these numbers.

Our work involving disease resistant pigs will require approximately 10 animals over 1 year. Six of these will be supplied to another license for a disease challenge experiment, with 4 genetically altered males maintained on this license for subsequent breeding purposes. If we choose to propagate these animals (eg if initial studies show significant promise) we will submit a license amendment which will inevitably increase these numbers.

Our work involving CDD in pigs will involve approximately 60 animals over 3 years. This will include approximately 10 animals under observation which will transfer from our previous license, 14 embryo donors, 6 embryo recipients and 30 piglets. This will be a sequential process in which we make preliminary observations of any disease phenotype in early cohorts of animals then refine our observation methodologies in subsequent animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All reagents used in the production of new GAAs are rigorously designed using computer programmes, tested in cultured cells and then tested in appropriate embryos ex vivo prior to use in animals.

All studies have been carefully considered and discussed by the scientific research team, institute vets and experienced animal staff. Where appropriate experiments are guided by previously published cell culture and animal data. As much of this work is discovery science the eventual outcome is often difficult to predict, and as such the numbers of animals predicted may require amendment (down or up) depending on the data generated.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For some of our projects the number of animals required is easy to predict based on previous experience and our knowledge of genetic inheritance (eg projects where we have already performed preliminary characterisation of a model and are now predominantly breeding to supply animals to other licenses). For others projects which are still in an early discovery phase we have made our best attempt to propose the minimum number of

animals required to achieve the scientific objectives. In addition to collecting samples directly relating to each project at necropsy we will also collect samples of other tissues which may be of value for future or related studies, biobanking these in order to optimize overall animal use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our sheep model of Batten disease is already well characterised, and does not develop disease when maintained in a heterozygous state (one allele of the disease-causing variant, and one allele of the wild-type variant). On this license the breeding population will be maintained in this state and then crossed to produced 50% heterozygous animals and 50% homozygous animals (either 2 copies of the disease-causing variant or 2 copies of the wild-type variant. All homozygous animals with disease causing alleles will be transferred to another license for translational studies or on rare occasions may be culled prior to disease developing. Animals on this license will therefore suffer no lasting harms.

Our sheep model investigating altered thermal tolerance is discovery science and we do not know what we will observe. A similar genetic variant in cattle results in shorter hair and a greater ability to cope with hot / humid conditions. We anticipate that we will observe a similar phenomenon in our sheep and do not anticipate that they will suffer any harms from the genetic alteration. We will collect small skin biopsies from these animals to assess the impact of the genetic alteration on skin morphology, and will supply appropriate anaesthesia and pain relief to minimise any suffering. This should be no more than moderate and transient.

Our pig model of hypertension will involve maintenance of animals and supply to another license for transgene induction studies. This will result in no harms on this license. We may additionally breed animals for subsequent studies.

Our pig model of SARS-CoV-2 will involve maintenance of animals and supply to another license for viral challenge studies. We may also collect small skin and / or lung biopsies from these animals to assess expression of the encoded ACE2 transgene, such that only appropriate animals are used for live viral challenge. Appropriate anaesthesia and pain relief will be used to minimise any suffering, which should be no more than moderate and transient.

Our GA pig model of disease resistance will involve collection of blood from which we will collect white blood cells for ex vivo viral challenge. Any suffering will be mild and transient. We may also collect a small skin biopsy from which we can culture fibroblast cells. Appropriate anaesthesia and pain relief will be used to minimise any suffering, which should be no more than moderate and transient. If initial studies are successful we may

also maintain and breed animals on this license for supply to another license for live viral challenge studies. No harms will be associated with this procedure on this license.
Our GA pig model of CDD may suffer harms as a direct result of the genetic alteration, and if such occurs is a required outcome for our further understanding of this disease in humans. Humans with reduced CDKL5 activity develop seizures, while rodent models involving the same genetic alteration do not. We do not currently understand why. In the first instance we will assess whether a pig model of this disease behaves more like the current rodent models or more like the human condition. In order to assess this we need to monitor animals to see if seizures develop. If seizures do not develop within the first year of life the experiment will be terminated and animals culled. If seizures do develop then we will next seek to accurately map disease progression, using a combination of behavioural observations and brain imaging (eg MRI), with collection of brains at necropsy for detailed analysis. We cannot collect this data without harms to the animals, but appropriate controls will be put in place to prevent suffering greater than is required for the scientific goals.

Why can’t you use animals that are less sentient?

Less sentient mammals tend to be smaller, and for our models that involve, or have the potential to involve translational studies the scale of the organism is important. In addition, for translational studies the physiology of sheep and pigs is more generally considered to be more similar to humans than are rodents.

For our CDD model, rodents do not recapitulate some of the more important disease characteristics observed in humans. We now seek to investigate if these characteristics are specific to humans or whether they are specific to species with larger brains.

Our model involving disease resistance is asking a species-specific question that cannot be answered in a less sentient species.

Our model investigating the potential for genetic alteration to impact thermal tolerance in an agriculturally valuable species similarly cannot be adequately answered in a less sentient species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We aim to minimise any stress or discomfort to experimental animals. Apart from under specific circumstances described elsewhere in this license animals will be group housed. Animal husbandry, surgery and anaesthesia will take place at our establishment's fully-equipped centre for large animal experiments.

Anaesthesia and surgery will be overseen by the highly qualified vets.

Daily welfare checks will be carried out by the NACWOs or trained PIL holders. Where a potential welfare issue is identified the frequency of these checks will be increased, including the use of 24 hour electronic recording where appropriate.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will refer to the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) during the planning stages and report our studies according to the ARRIVE 2010 guidelines (Animal Research: Reporting of In Vivo Experiments). The principles laid out in these resources will enable us to design and conduct the experiment in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Animal welfare will be prioritized throughout the study by incorporating all permissible refinements identified by the establishment's Named Veterinary Surgeon and other veterinary colleagues.

The NC3Rs website (https://www.nc3rs.org.uk/) will also be consulted and full advantage taken of the annual seminar day organized by the establishment to identify changes in best practice and methods to improve animal welfare.

Any adaptations will take place following discussions with the NVS and NACWOs before incorporating them into the study plan.

208. Investigating Risk Factors and Therapies for Dementia

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Alzheimer's disease, Inflammation, Traumatic brain injury, Therapy, Hearing loss

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged
Rats	adult, juvenile, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The vast majority of dementia cases are likely to be initiated by environmental risk factors and many of these risks factors involve an increase in inflammatory processes in the brain; for example, a previous history of head injury, the use of anti-depressant drugs, hearing loss, etc. Using animal models, our aim is to investigate how these risk factors affect the development of dementia symptoms and if they can be mitigated by treatments.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Alzheimer's disease (AD) is the most common neurodegenerative disorder. The cardinal symptoms of Alzheimer's disease in the early stages are memory loss and modest changes in behaviour. These symptoms slowly progress with additional deterioration of the patient's executive function, language and visuo-spatial abilities, with the patient gradually declining into total dementia and losing their autonomy. The average survival time from the point of diagnosis is 5.7 years for women and 4.2 years for men.

There are more than 50 million cases of dementia worldwide. In the UK, the government spends over 26 billion pounds a year on care for dementia patients, so it is a social and economic problem as well. Unfortunately, current treatments for AD only provide short-term symptomatic relief rather than disease-modifying benefits.

Our studies will provide insight into the causes of dementia and some of the early pathological processes in the Alzheimer brain and will add additional impetus to new treatments or prophylactic action to stop the progress of the disease.

What outputs do you think you will see at the end of this project?

The results of this work will allow the identification of new targets (at medium term) for therapeutic intervention and will help to understand the causes of Alzheimer's Disease.

In addition, the data obtained in this project will result in new publications in the field (short term).

These studies will lead to potential new treatments or strategies to stop the disease progression or ways to prevent the disease. The development of new treatments and interventions will be long term, because they need to be trialed in humans and this takes many years.

Who or what will benefit from these outputs, and how?

In this project, we are investigating the way that risks factors affect the progress of the pathology and symptoms of dementia and how the potential new treatments work (what are the effects).

The discovery of the potential causes of AD and new treatments will benefit a great part of the population, because in the world there are more than 50 million cases of AD. In the UK, the government spends over 26 billion pounds a year on care for dementia patients, so it is a social and economic problem as well. In addition, the results could be extrapolated to other neurological disorders that have common mechanisms with AD.

The results will also benefit the scientific community (both basic scientists and clinicians), by establishing the contribution of neuroinflammation in models of chronic neurodegeneration and the use of new technologies (such as imaging and non-invasive brain stimulation) to understand the causes and potential new therapies of the disease, which could also be extrapolated to other neurodegenerative disorders.

How will you look to maximise the outputs of this work?

We will disseminate the results of this research through publication in peer-reviewed journals, presentations, seminars and conferences.

We are collaborating with other research groups in our Institution on this project with expertise in electrophysiology and imaging.

Furthermore, we collaborate with clinicians in order to move the therapies into trials, using our results as proof of concept.

Species and numbers of animals expected to be used

- Mice: 2000
- Rats: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Within the past 3 decades, our understanding of the pathogenic mechanisms in Alzheimer's disease (AD) has dramatically advanced because of the development of transgenic mouse and rat models that recapitulate the key pathological and behavioral symptoms of the disease. These models have allowed investigators to test detailed questions about how pathology and memory impairment develop and to evaluate potential therapies that could slow down the development of this disease. We usually use

mouse lines, because they are more models of dementia available in mice (animals overexpressing or lacking particular genes involved in dementia). However, we also use rats, particularly for traumatic brain injury, because their brains are bigger (ideal for imaging studies) and their behaviour is more complex.

The patients and models of dementia usually develop symptoms at old age and this is why we need to use older animals. In some cases, we run longitudinal studies (studies over time) at different ages to understand how the disease progresses and how treatments may work better at one particular age or stage of the disease.

Typically, what will be done to an animal used in your project?

The animals will undergo treatments (which sometimes will be delivered directly into the brain), behavioral tests to measure changes in memory, in vivo imaging (scanning) to determine changes in brain structure, electrophysiology (placement of electrodes to record the activity of the neurons) to investigate alterations in neuronal function and blood sampling to measure biomarkers, although some of these procedures are optional. Also animals will have imaging windows in the skull to perform life imaging using 2-photon microscopy (a fluorescence imaging technique that allows imaging of living tissue).

To investigate the effect of hearing loss, ear plugs will be placed in the ears of the animals and will be removed to determine whether the effects on memory are reversed.

In addition, we will use models of traumatic brain injury, which reproduce the pathology seen in humans and develop memory and neuronal loss and is a risk factor for dementia. The animals that will be subject to traumatic brain injury will undergo some procedures,

such as the closed head injury model (which involves the use of an impactor hitting the top of the skull), which will require surgery and anesthetics. Another model that will be used will be the blast model, which mimics the effects of explosions.

The behavioural tests take place over several days, but the rest of the procedures are short and last few hours. The treatments could last from one day until one month.

What are the expected impacts and/or adverse effects for the animals during your project?

Animal models of dementia suffer memory loss, but this does not cause pain. The behavioural tests used have a mild level of severity. In the case of the fear conditioning test there is some mild pain. Some models, such as the animals with tau pathology (which occurs in AD and frontotemporal dementia) can suffer motor symptoms as they age (as the clinical symptoms of patients with frontotemporal dementia), which include slow movements or loss of balance and coordination. From ages of 13-14 months old, symptoms include paralysis of the hind limbs, but the animals will be killed before this occurs. Animals will be monitored (both visual examination and weight measurements) and we do not expect that they will live longer than 1 year old, when they develop these adverse symptoms.

Traumatic brain injury (TBI) models may also suffer minor memory and motor impairments, because they mimic the same symptoms of patients that suffered head injury. The closed head injury model and

the blast model have a moderate level of severity. The closed head injury model that we use comprises the opening of the skin and the controlled impact on top of the skull, which is done under anaesthesia. The animals also receive analgesics. In our experience with this model, we observe that the animals recover very fast after the surgery and do not have overt symptoms, unless we carry out behavioural tests. In addition, the brain does not have the tissue loss observed for the same model with craniotomy. Furthermore, we have not observed any death following the impact step, only few deaths under non-recovery anaesthesia. This model is useful to mimic what happens when a football player hits a ball or during boxing.

The blast model reproduces what happens in many soldiers that have suffered explosions. It is induced by the use of blast pressure waves from a shock tube, affecting only the head of the animal, under anaesthesia and analgesia. This model also does not display evident changes on the animal’s brain or behaviour; even when brain scans are carried out, the structure is very similar to a control animal. We are investigating if there are changes at more subtle level (using electrophysiology) or molecular changes that can lead to the animals developing alterations long term post-injury.

For the animals with hearing loss we do not expect any adverse effect, just a bit of discomfort. Occasionally, the implants may become dislodged or fall out.

All animals will be closely monitored after any surgery (both visual examination and weight measurements), and additional analgesia and/or antibiotics provided as necessary.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?
In general and based in our previous work, the level of severity will be mild (91%) or moderate (9%) in mice and mild (21%) or moderate (60%) or non-recovery (19%) in rats.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mouse and rat models of AD reproduce in great extend the brain pathology in humans. Humans with AD have deposition of certain proteins in the brain, including neuritic plaques (aggregates of misfolded proteins (amyloid-β peptides) that form in the spaces between nerve cells) and neurofibrillary tangles (aggregates of misfolded proteins (tau proteins) that form inside nerve cells).

In addition, these animal models show vascular alterations, increased inflammation and memory loss and allow us to understand the pathogenesis of AD, which cannot be detected or measured in cells models (in vitro). There are some invertebrate models of AD, but the brain anatomy is not similar to humans and the behavioral abnormalities of AD are difficult to address.

Which non-animal alternatives did you consider for use in this project?

We do initial work with cell lines, including neuroblastoma cells and glial cell lines, before we move to animal work.

The cells are used to determine whether certain drug or protein (which we can over-express or knockdown) is making any effect on the levels of soluble Amyloid-β, inflammatory markers and the potential cell toxicity, before we undertake an animal work. We also use the cells to investigate the effect of the drug in a particular cell type, using a less complex model, since brains contain many different cell types. However, it is still possible that concentrations that do not kill the cells in cell culture may have some toxic effects in animals, this is why we still need to carry out pilot work in small number of animals.

In addition, we are in collaboration with a group in Engineering that creates computational models of traumatic brain injury. Computational biomechanics models can be used to guide the design of new experiments without sacrificing a large number of animals. Our recent experimental and computational studies have shown correlation between mechanical forces and different pathologies, e.g. oedema, neuronal loss, axonal damage and vascular injury.

Why were they not suitable?

Cell models do not show the relationship between different cell types in the brain and do not allow monitoring behavioral changes or alterations in brain anatomy and amyloid deposition.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our projected animal numbers reflect the number necessary to achieve the scientific objectives outlined in the programme of work described in our application.

These numbers are based on statistical analysis and in our previous experience. For behavioral analysis, we need 10-12 animals per group, while for other types of studies, such as protein investigation, it is enough with 5-7 animals per group.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use good experimental design using tools such as the NC3Rs experimental design assistant and ensure they are appropriately powered to address your scientific objectives, according to software that we are using for power calculations called GPower version 3.1.

The number of animals are minimized by doing the experiments 1) using brain cell cultures to determine whether we have a positive result 2) using cultures of brain slices from pups; 3) a short number of wild-type (non-transgenic) animals for treatment.

We will be conducting the experiments in order to be able to publish according to the ARRIVE guidelines and will use randomization, blinding, ethical statements, experimental procedures, details of experimental animals (number, strains, side effects), sample size, husbandry where appropriate and avoid biases.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will be conducting pilot studies; initially we will be using no more than 3 animals per group of AD transgenic mice for testing novel compounds, typically at two or three dose levels around the expected pharmacological dose. For comparisons using larger groups, group size will be estimated by statistical analysis.

In addition, we will be conducting the genotyping of the mice prior weaning so we will keep only the mice that we need for the experiments.

We usually extract the brains of the animals that we do not need for the study and keep them frozen for future studies or we share them with other groups.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse and rat models of dementia are extremely valuable tools in dementia research, in particular Alzheimer's disease (AD), which is the most common form of dementia. The two identifiable hallmarks of AD, which involve the deposition of proteins that aggregate (forming amyloid plaques and neurofibrillary tangles (NFT)) in the brain, have been reproduced in rodent models. These animals also show memory loss as they get old. In addition, previous work in these models has demonstrated that increased inflammatory processes in the brain affects the generation of amyloid plaques and NFTs as

well neuronal death. Importantly, a correlation of cognitive performance with the protein/plaque pathology has been demonstrated in behavioral studies. The majority of the protocols used are aimed to understand what causes dementia, examining changes in brain function (by behavioural tests measuring memory issues and recordings of neurons) and pathology (using imaging techniques), as well as how potential therapies could reverse these changes. These procedures are minimally invasive or moderate and therefore longitudinal studies in the same subject may be performed.

In this new PPL, we are investigating the link between traumatic brain injury and dementia, as it is an important risk factor. We use two models of traumatic brain injury, both with moderate severity. The close head injury model comprises the opening of the skin and the controlled impact on top of the skull (under anasthesia and analgesia) and does not require removal of the portion of skull overlying the area of the brain to be injured. Therefore, it is a more translational model and causes less pain and distress, compared with other models requiring craniotomy, which are more severe. In our experience with this model, we observe that the animals recover very fast after the surgery and do not have overt symptoms, unless we carry out behavioural tests. In addition, the brain does not have the tissue loss observed for the same model with craniotomy. Furthermore, we have not observed any death following the impact step, only few deaths under non-recovery anaesthesia. This model is useful to mimic what happens when a football player hits a ball or during boxing.

The blast model of TBI (the non-invasive controlled pressure wave model) involves the use of a pressure blast wave released by a shock tube under anesthesia. This model is not expected to cause overt injury to the animal and does not display evident changes on the animal’s brain or behaviour; even when brain scans are carried out, the structure is very similar to a control animal. We are investigating if there are changes at more subtle level (using electrophysiology) or molecular changes that can lead to the animals developing alterations long term post-injury, as it happens in many soldiers that have suffered explosions. The classification of the Blast model severity is moderate.

In addition, another potential risk factor for dementia is hearing loss. To investigate this. we will promote reversible hearing loss to animals using special earplugs, in order to determine how hearing loss leads to dementia. We will determine whether the animals

change their behaviour when they do not hear well and if this affects areas of the brain involved in hearing and in dementia. Importantly, we will explore whether removing the ear plugs reverses the effects and improve memory and brain function. We will minimize the suffering by providing anesthesia and analgesia.

Why can’t you use animals that are less sentient?

Non-protected animals (e.g. invertebrates such as insects, decapods, nematodes) or less sentient animals (eg zebrafish) are further apart from human biology. Furthermore, we consider that is ethically superior to use adult animals. Brain anatomy from invertebrates is very different compared with humans and their behaviour is not complex and cannot be compared with humans. Insects and fish do not develop amyloid plaques and they do not show sign of senescence, since they only live for few days.

We will be doing some experiments in organotypic cultures (cultures of brain slices from pups), in some cases.

For some electrophysiological analysis (which involve the recordings of neuronal activity) and for certain imaging procedures animals will be terminally anesthetized. However, some of the experiments or procedures cannot take place under non-recovery anaesthesia, because the animals need to be awake, for instance to perform behavioural tests.

We also need to follow the progression of the disease over time or to determine if a treatment is effective, which make take some months.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will be following close monitoring (by visual inspection and measurements of the weight) in animals after moderate procedures (such as surgery) or the administration of certain pro-inflammatory substances and will increase pain management. For behavioral tests, we will be handling the animals some days before the tests. Handling of animals is intended to acclimatize them so that when they are subsequently used in behaviour tests they will be used to the researcher and this could potentially decrease stress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the ARRIVE guidelines and will use randomization, blinding, ethical statements, experimental procedures, details of experimental animals (number, strains, side effects), sample size, husbandry where appropriate and avoid biases.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The team will get actively involved and learn from other researchers both within the College and externally as well as the staff of the animal facilities and AWERB. We receive updates and information by e-mail on sessions and activities to improve the 3Rs.

209. Ligament Repair

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

ligament, repair

Animal types	Life stages
Sheep	adult
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this research project is to develop and commercialise fixation devices for ligament repair and innovative methods of repair, which address the unmet clinical need to improve soft tissue graft fixation, integration and therefore accelerate healing response, rehabilitation and return to normal activities.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The output of the project will have commercial value and will enable to offer improved technologies to enhance and accelerate the repair of ligaments.

What outputs do you think you will see at the end of this project?

The primary purpose of this project is to improve the experience of patients undergoing ligament surgery. It also aims to reduce costs for the Health Service due to shorter stays in hospital. Currently there is a large unmet need for developing technologies and products to enhance ligament surgeries that reduces pain and suffering to patients and decreasing the financial burden on the healthcare system. These technologies and products will help patients worldwide to regain normal lives in terms of their ability to carry out everyday functions which had been prevented either by degenerative joint disease or by trauma. The economic benefits would include fewer days lost at work, fewer hospital days and reduced care costs.

Who or what will benefit from these outputs, and how?

SHORT-TERM BENEFITS (0-3yrs): Although, the current set of in-vivo models have shown to translate clinically, there is still an opportunity to expand and increase the clinical relevance of the models available. These new models will in turn, facilitate the development of innovative products, which will improve patient outcomes. The project will attempt to develop new experimental models of ligament repair, which will help advance the frontiers of science in this area for the benefit of clinicians appraising preclinical ligament repair studies. It will also allow us to investigate new indications for some of their strategic acquisitions, which could be used to enhance ligament repair. These therapeutics will be tested using existing ligament repair models developed under previous licences.

LONG-TERM BENEFITS (5-10 years): The project will also provide long term benefits for patients suffering from acute and chronic full-thickness tears will benefit in the short-term as they should be able to return to a day-to-day life much quicker and experience less pain. Long-term benefits would be the economic benefits which include fewer days lost at work, fewer hospital days and reduced care costs.

How will you look to maximise the outputs of this work?

The outputs of the project will be fully exploited through a number of different mechanisms. For example; (a) product support data, (b) pre-clinical regulatory support data, (c) conference abstracts and posters, (d) scientific publications outlining new experimental models of bone ingrowth. Model development may also involve the support of either academics or clinicians, which will assist with knowledge transfer.

Species and numbers of animals expected to be used

- Sheep: 1000
- Rabbits: 50

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Sheep have been selected as a large animal experimental model for protocols: 1 Partial patellar tendon soft tissue-bone reattachment, 2 Patellar tendon reattachment (whole tendon) 3 long digital extensor (LDE) tendon reattachment 5 Soft tissue (tendon) ACL reconstruction 6 Stabilised ACL implantation model 7 Chronic partial patellar tendon soft tissue-bone reattachment 8 Acute infraspinatus tendon (IT) model for soft tissue-bone reattachment 9 Chronic infraspinatus tendon (IT) model for soft tissue-bone reattachment, given that their anatomical size, histomorphological characteristics, skeletal dimensions and ease of supply. Skeletally mature sheep are preferred over juvenile and aged animals in this licence. Rabbits have been selected as a large animal experimental model for protocol 4 Long digital extensor (LDE) tendon reattachment. Small animals such as rats and mice would be more difficult to use as an animal model of ligament repair given the practicality in designing customised implants, small bone volume and differences in histological and anatomical relevances to human.

Typically, what will be done to an animal used in your project?

The project will involve the creation of a bone defect, surgical transection and/or reattachment of the ligament, which may lead to some degree of discomfort following surgery, although this will be reduced by the use of a minimally invasive technique. The maximum level of severity will be moderate. The duration of the surgical procedure should not exceed 2 hours. The animals will be humanely euthanised at the end of the protocols.

What are the expected impacts and/or adverse effects for the animals during your project?

The surgical procedures detailed in this licence will cause mild or moderate post-operative discomfort including pain, lack of appetite, lameness, swelling, which will be controlled by analgesics and refinements made from our previous experience. The estimated duration of these effects on an animal is up to 8 weeks for swelling and up to 2 weeks for other parameters. Antibiotics will be used to prevent any infection. Any discomfort will be minimised with the use of appropriate pain relief. At the end of the studies the animals will be humanely euthanised. In addition any animal showing severe signs of suffering whilst on study (e.g. excessive weight loss, signs of uncontrolled pain, significant lameness) will be humanely euthanised.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For all protocols on this licence, it is expected that up to 100% of animals (both sheep and rabbits) will experience moderate harm in work under this protocol.

Due to the enforcement of humane endpoints in each protocol, it is highly unlikely that any animals will suffer any severe harm.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals will only be used where there are no alternatives to the use of animals in order to answer the questions that the project requires.

Ligament repair is a complex, well-orchestrated physiological process of bone formation involving signaling cascades and cellular repair mechanisms that cannot be studied by in-vitro cell culture studies alone where these complex interaction are impossible to truly replicate. Consequently, ligament repair can only be demonstrated with the help of animal models, i.e. no in-vitro methods such as computer simulations or cell cultures can mimic the complexity of an in-vivo environment sufficiently or predict clinical efficacy. The in-vivo models developed under this licence tailored for studying ligament repair will attempt to reflect the biomechanics and the physiology of the particular clinical scenario in humans. There is currently no suitable in vitro alternative to the animal model for assessment of ligament repair.

In-vivo models will also be required when in-vitro systems cannot provide a reproducible approximation of the real-life in-vivo or clinical setting, e.g. the kinetics of delivery and distribution of drugs or bioactive factors; the biocompatibility and degradation properties of implant materials.

Regulatory authorities such as that FDA will also require the testing of novel bone repair therapies in both a small and large animal model before accepting an agent for clinical trials to ensure clinical translation in bone tissue engineering and regenerative medicine through assessment of appropriate efficacy and safety endpoints.

Which non-animal alternatives did you consider for use in this project?

In-vitro cell culture models will be used for studying bone and tendon mechanobiology in 2D by applying controlled mechanical stimuli to relevant cell lines and also to establish safety of any technology prior to any in-vivo experimentation. Collectively, these tests will be used for screening out some of the early stage technologies.

Why were they not suitable?

These preliminary tests will be used to screen out novel technologies that lack supportive data. However, in order to provide a sound basis for making determinations about reasonable safety and efficacy, animal models will be required to provide accurate information about how a medical intervention will perform in a human clinical trial. Furthermore, regulatory authorities such as the FDA and MHRA will require evidence of in-vivo safety and efficacy data prior to clinical evaluation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This licence propose to use sheep and rabbit models and the number of animals, based on the number used in previous licences, will not exceed 1050.

The number of animals per species required will be determined from a number of sources:

The experiences gained from previous licences.

ISO standards (ISO 10993)

Our statisticians input at the planning stage of the in-vivo studies to advise on study design, post live phase analysis and to determine the minimum number of animals required to provide sufficient likelihood of a meaningful outcome.
Previous studies or studies reported in the literature will be used to provide variability data to aid this process, or pilot studies will be conducted to generate such data. This will reduce the numbers of animals used in total without compromising the data/information obtained.

Our organisation's AWERB, which will assess all protocols and experimental design prior to the start to ensure a minimum number of animals are used to the meet the study objectives. Our AWERB review every proposed study in addition to reviewing the protocols proposed in this licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our organization will adopt multiple strategies that will help ensure that the fewest number of animals will be used in the research to address the scientific questions outlined in the project.

SUBJECT VARIABILITY: Variability will also be reduced through the procurement of animals of consistent breed, sex, age and weight ranges and through application of animal acceptance criteria for each study.

BIOSTATISTICS/POWER ANALYSIS: Statisticians will be consulted in the planning stage of the in-vivo studies to determine the minimum number of animals required to for statistical analysis and to answer a scientific question being asked. This will reduce the numbers of animals used in total without compromising the data/information obtained. Consultation with a statistician will comprise setting clear study objectives, and ensuring appropriate output measures are collected and analysed using appropriate statistical methods. Sample sizes will be determined based upon the needs of the study which may be tailored for either welfare, pilot, validation or efficacy/non-inferiority/equivalence. Where powered, historical data will be used to determine the appropriate sample size to achieve the required study power. In order to minimise animal numbers used across the project every effort will be made to test as many candidates as possible in a single experiment against a single control group. Typically, welfare studies to assess new procedures or technologies under this licence will consist of no more than four animals. Where there is

no adequate data to power a study, a pilot will be used to gather sufficient data to design a definitive study. Typically, these will be designed to provide a minimum of 10 degrees of freedom to estimate the error. For example, a study with two groups would have a sample size of 6 per group. Where historical information is available the study size will be determined by the minimum numbers required to provide sufficient power (at least 80%) to achieve the desired outcome.

To further minimise numbers, where possible, one sided statistical tests will be used. The objectives dependent on the outcome measures may be to show superiority to a control, non-inferiority to a predicate or gather device performance data. Sources of variability will be controlled by giving careful thought to potential sources of error, bias and variation in measurements, and making every effort made to minimise them. This will include (a) using well-characterised implants that are within specification, (b) defining the success criteria of the study, (c) adopting a consistent surgical technique across the studies, (d) providing adequate time for acclimatization, (e) training of staff, (f) blinding observers and participants to the study hypothesis, and (g) adopting a randomisation schedule in order to reduce bias and interference caused by irrelevant variables.

LOCAL ETHICS (APPROPRIATE EXPERIMENTAL DESIGN): Our organisation's AWERB will assess all protocols and experimental design prior to the start to ensure a minimum number of animals are used to the meet the study objectives. Our AWERB review every proposed study in addition to reviewing the protocols proposed in this licence.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

COMPUTER SIMULATIONS: Computer generated models of the anatomy will be generated from CT scans of isolated limbs to create 3D models that can mimic functions of physiology and help determine safe corridors for surgical implantation. These models will also be used to develop customized implants reducing the risk of any surgical complications.

In-Vitro Testing: In-vitro cell culture models will be used for studying bone and tendon mechanobiology in 2D by applying controlled mechanical stimuli to relevant cell lines and also to establish safety of any technology prior to any in-vivo experimentation

QUALITY ANIMALS/VETERINARY CARE/PRE-SCREENING: Radiological templating of the animal prior to surgery will help screen out animals that are deemed to be unsuitable for surgery due to either health reasons or unsuitable anatomy. The images collected can also be segmented to create 3D models to assist with implant development. The loss of animals can also be minimized by providing good post-operative care, avoiding unintended breeding, and planning ahead so that the appropriate number of animals needed for the studies are ordered and/or bred.

PILOT WELFARE STUDIES: Pilot studies can be used to estimate variability and evaluate procedures and effects. Where the primary output measure of the pilot study is to establish acceptable welfare of animals subject to either new procedures or technologies under this licence, no more than four animals will be used.

PILOT "POWERING" STUDIES: Where there is no adequate data to power a study, a pilot will be used to gather sufficient data to design a definitive study. Typically, these will be designed to provide a minimum of 10 degrees of freedom to estimate the error. For

example, a study with two groups would have a sample size of 6 per group. Where historical information is available the study size will be determined by the minimum numbers required to provide sufficient power (at least 80%) to achieve the desired outcome.

APPROPRIATE USE OF ENDPOINTS - TISSUE SHARING: Where possible, harvested tissues will be recycled for multiple testing, e.g. blood draws, biopsies, CT, histology and biomechanical testing.

SHARING ANIMALS: For instance, animals euthanized by one investigator can provide tissue for use by another investigator on another licence or protocol.

NEW INSTRUMENTATION AND TECHNIQUES: Using new instrumentation or innovative techniques that can improve precision can reduce the number of animals needed for a study. This has the added benefit of also being a refinement technique for the protocol.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

A thorough investigation into the most relevant species for simulating ligament repair have been obtained from the knowledge gained from previous project licences.

All models are already developed by our facility. These models and methods have shown that they cause the least amount of pain, suffering and distress to the animals.

Most of the protocols listed in this licence will utilise sheep as this species has bones of the size that are compatible with human implants and surgical techniques. Our facility have accrued a great deal of experience with these animal species from previous licences. This experience has led to refinements in surgical technique, analgesic regimes and post-surgical care. Gait analysis has been used successfully to monitor recovery after surgery and this analysis has been used to improve post-surgical care. The long digital extenson repair protocol allows to utilise rabbits as well as sheep.

Why can’t you use animals that are less sentient?

Live mammalian vertebrates are required that closely mimic the ligament repair pathways and human skeletal system as much as possible to ensure that any data generated can be translated to the clinical situation.

Small animals such as rats and mice would be difficult to use as an animal model of ligament repair given the practicality in designing customised implants due to small bone volume and differences in histological and anatomical relevance to human.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Refinement applies to all aspects of animal use, from housing and husbandry to the scientific procedures performed on them. Continued investigation into animal refinement will be sought through several sources, e.g. (a) careful choice of animal model, (b) adoption of a multi-disciplinary team with expertise in animal husbandry, housing and care, veterinary science, pain management, engineering and project management, (c) improvements in animal procurement, transportation and quarantine, (d) improvements in animal husbandry such as training of animals and group housing to habituate animals to study procedures to minimise any distress, (e) implementation of housing, e.g. micro- and macroenvironment, (f) increased monitoring and surveillance, (g) refinement in surgical techniques, e.g. minimal invasive surgery that minimize animal pain and distress, (h) appropriate anaesthesia, analgesia and sedatives to minimise pain and (i) post-operative care/recovery and (j) pain management (anesthesia, analgesia, drug pumps).
SOPs will also be regularly updated and documented within our Quality Management System, which is accredited to ISO9001. Staff training will also be made available through attending courses and conferences and integrating with key opinion leaders.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Best practice approaches will be used to enhance animal well-being, minimize or avoid pain and distress, and reduce the number of animals required to obtain the desired research objectives. Best practice on animal care and husbandry will also be achieved through several sources including (a) our facility's Animal Welfare and Ethical Review Body (AWERB) with an advisory function on ethical matters, (b) UK Home Office guidelines on Animal Testing and Research https://www.gov.uk/guidance/research-and-testing-using-animals, (c) NC3Rs, which is a UK-based scientific organisation dedicated to replacing, refining and reducing the use of animals in research and testing (the 3Rs) https://www.nc3rs.org.uk and (d) consultation of the Guide for the Care and Use of Laboratory Animals (Source: National Research Council of the National Academy of Sciences 2011).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The subject matter experts that are employed within the animal facility will engage in continuous professional development that will ensure best practices in pharmacology, radiography, animal husbandry and welfare are regularly adopted during the lifetime of the project.

210. The mechanisms underpinning ‘steroid’ (glucocorticoid) development of obesity and diabetes

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Steroids, Glucocorticoids, Diabetes, Obesity, Brain

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Synthetic glucocorticoids, commonly called steroids, are used as medicines to treat a wide range of illnesses such as asthma, rheumatoid arthritis, and multiple sclerosis. Although well tolerated, long term, high dose use can lead to side effects including obesity and diabetes. Therefore, the aim of this study is to identify how longer term use of glucocorticoids acts in specific parts of the brain to increase food intake, and to alter processes in the body leading to high glucose and diabetes. Our long term aim is to find alternative synthetic glucocorticoids or alternative ways of administering them to reduce the associated obesity and diabetes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Glucocorticoids (steroids) are a widely used class of medicines. They are anti-inflammatory and used to treat diseases such as asthma, arthritis and autoimmune conditions such as multiple sclerosis as well as in the treatment of cancer. With long term, high dose use, patients can develop side effects similar to those seen in the metabolic syndrome, a cluster of illnesses associated with obesity and diabetes. These include weight gain, especially around the waist, type 2 diabetes, and fatty liver disease. These side effects can limit the use of an otherwise good and inexpensive medication. In this body of work, we want to understand how the glucocorticoids cause the side effects. By understanding this, it might be possible to find other glucocorticoids that don't cause the side-effects or medicines to be given with the glucocorticoids that decrease the side effects. This would mean that more patients could be treated with these medicines.

What outputs do you think you will see at the end of this project?

At the end of the project we will have gained new information about how glucocorticoid treatment causes side effects such as obesity and diabetes. This information will be shared in research papers and with other scientists and doctors at conferences. If we can understand how the side effects develop then we, with doctors and the pharmaceutical industry, will be able to design new types of glucocorticoids which don't cause the problems or different treatments given with the glucocorticoids to reduce the side effects.

Who or what will benefit from these outputs, and how?

The main beneficiary of this work will be patients treated with glucocorticoids and their doctors who are able to prescribe them with reduced worries about side effects. The NHS will also benefit, as glucocorticoids are cheap medicines and will be able to be used more widely. Additionally, the costs of treating the side effects such as diabetes are also high, so these may be reduced if the side effects of glucocorticoids are reduced. The scientific community will also benefit from the outputs generated in this project, as we will be increasing the knowledge of how these steroids work.

How will you look to maximise the outputs of this work?

Previously, we have collaborated with groups at other universities, with pharmaceutical companies, and with clinical endocrinologists who see patients with these side effects. Therefore, we are well placed to contact those groups of people who can use the outputs of our studies most effectively. These collaborations have not only been for exchange of knowledge, but also to learn the most refined way of carrying out the experiments. We will present our early data at conferences, so fellow scientists and doctors can comment on the experiments while they are progressing. This will ensure that the correct studies are being carried out. We will also publish our data, whether successful or not, in open access scientific journals as well as sharing our new publications on social media platforms to increase the visibility. All raw data will be made available upon request.

Species and numbers of animals expected to be used

- Mice: 10500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

For our research we use an adult mouse model, which is treated with glucocorticoids (steroids). This model is able to mimic the side effects of the treatment that are seen in humans. Also, the pathways in the development of these side effects of obesity, diabetes and fatty liver disease are thought to be similar between humans and mice. In addition, we are looking at actions in the brain so we need to collect brain tissue and we look in mice bred with certain pathways removed, making these animals the best model for our investigations.

Typically, what will be done to an animal used in your project?

Most mice in this project will be treated with glucocorticoids to look at the side effects of diabetes and obesity. Typically, we give this treatment in their drinking water to reduce the stress of handling and the short term pain of an injection. They will be weighed frequently and depending on what we are looking at they may have a glucose tolerance test, where they will be fasted before they are injected with glucose and small blood samples taken from the tail. Alternatively, they may have their metabolic rate measured by placing them in special cages that can measure this. We also may look at how much fat they have using a type of MRI scan, but as it doesn't give an image, they don't need to stay as still. Before the end of the study, we take blood samples from the tail to measure things such as stress hormones and insulin. Many of our protocols look at changes in factors associated with appetite and feeding in the brain, but these measurements are made after the study finishes.

Some studies will be carried out where we need to give drugs directly into the brain. For these studies, mice will undergo a short surgery with a quick recovery anaesthetic and a device will be inserted, so these drugs can be directly administered to the brain when the mice are awake and freely running around. During and after surgery, mice will be given appropriate pain relief, kept warm and given soft food to help them recover as quickly as possible.

What are the expected impacts and/or adverse effects for the animals during your project?

With glucocorticoid treatment the mice should gain weight and become overweight or obese. The treatment period for these studies are 4 weeks at their longest, so the animals are just at the start of developing diabetes. Some will develop diabetes and therefore drink more and urinate more, but we will change their bedding more frequently to account for this. As the measurement of food intake is important for our research, animals will often be in a cage on their own, which can disrupt their normal behaviour, but we provide them with tubes to play and hide in and additional bedding and chew sticks to reduce the impact of this. The total length of time where the animals would be housed alone is generally 7 weeks (1 week to acclimatise, 2 weeks to take normal measurement and then 4 weeks on treatment). A very few studies may want to investigate genetic changes related to glucocorticoids in mice and then mice may need to be housed alone for up to 6 months.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most animals will have a mild experience. They will eat more and will gain weight, but not to the point that it impacts their welfare. There could be transient pain from injections or blood sampling. There may be a mild stress effect of social isolation.
The animal having surgery will have a moderate severity experience. They will be given quick recovery anaesthetics to reduce the impact of the anaesthetic, painkillers to reduce any pain and will be kept warm until fully recovered. Mice tend to recover quickly from this surgery.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We look at the effects of glucocorticoids (steroids) on the whole of the body. We mainly give these to the animals in their drinking water, which is similar to patients swallowing tablets, and we look at their effects on multiple organs in the body, such as fat, liver and muscle. It is not possible to mimic these effects in dishes of cells as you cannot look at the overall effect on the body.

Additionally, we look at how glucocorticoids acting in the brain then signal to the other organs in the body. As we are looking at how one organ in the body controls others, a whole body system is needed for this.

We have found from our previous studies that mice respond similarly to patients when given these steroids, so they are used in our studies.

Which non-animal alternatives did you consider for use in this project?

A potential alternative is to work on neurons growing in culture. However, these neurons do not survive and do not develop into cell lines. There is a technique for adapting them to grow continuously which is complex but possible, and this offers a way of addressing specific questions about the neurons that regulate food intake. Several years ago, we obtained immortalised hypothalamic neuronal cell lines from a collaborator to determine if they produced a key neuropeptide regulating food intake. Despite a lot of effort in culturing the cells, we were unable to get these neuronal cell lines to synthesise this neuropeptide. We subsequently worked with another collaborator who uses embryonic stem (ES) cells differentiated into neurons and studied the production of these neuropeptides. As the neuropeptides are a glucocorticoid (steroid) target in the brain, we will be able to assess the effects of chronic glucocorticoid treatment on these peptides in the ES cells. This

would complement the *in vivo* studies where we consider the effects of glucocorticoids on food intake.

Why were they not suitable?

As stated above, neurons which were adapted to grow continuously did not produce the neuropeptides, but we have subsequently collaborated with a research group using ES cells which could provide key information on the effects of chronic glucocorticoid treatment. However, there is growing evidence that these neurons are quite heterogeneous in the brain and they act at several different target neuronal populations which would be impossible to mimic *in vitro*.

In addition, to prove that glucocorticoids are acting via these specific neuropeptides to modify food intake, body weight and peripheral metabolism, we will still need to do these studies *in vivo*. We also need to study the effects of glucocorticoids on multiple body tissues such as liver, adipose tissue and muscle at the same time, to examine the molecular changes underpinning the side effects in patients treated with these medicines.

Therefore, single cell types will not inform us of the overall effects on the body.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Generally, we use the data from previous studies to help us estimate the number of animals required for an experiment. This allows us to use the minimum number of animals to give us a reliable answer to the question. Where it is a completely new experiment, we will use a small group size at the start, so we can monitor how many animals we will need.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To design the experiments we use the NC3Rs experimental design assistant to get the best study design and to use the correct number of animals. At the end of the study we take organs from the animals that we might not necessarily need at the moment, but so we wouldn’t need to run another study in the future if we need to look at the effects of treatment that organ.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We breed the minimum number of animals possible for our projects that will give us the best experimental results. When we start new studies, we carry out pilot studies to understand how the animals will react and to help us estimate the number of animals we will need. At the end of the study, we take as many tissues and organs as possible, to

minimise the need to run later studies, we also share the tissues from some studies with other groups, so they don't need to run their own experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This licence application only uses mice, the least sentient animal possible for our studies. Mice are used as they give similar responses to glucocorticoids (steroids) as is seen in patients treated with these medicines. We mostly give the glucocorticoids in the drinking water, so the animals don't need to be handled and stressed as much and this approach reduces the need for multiple injections. None of the mice with genetic changes show any side effects to these gene changes.

Why can't you use animals that are less sentient?

We have investigated zebrafish models and there are some good examples of novel data describing how glucocorticoids act within the cells. There has also been information on how glucocorticoids control development of the zebrafish. However, the systems are too limited to be used to study whether the glucocorticoids are acting in the brain or directly in organs in the body that regulate metabolism. In addition, there seem to be key differences in zebrafish in the expression of enzymes that activate/inactivate glucocorticoids. We have also investigated what is known in zebrafish about the melanocortin system, which is a target of glucocorticoids that this project addresses. Unfortunately, previous work has only studied a rudimentary peptide system in another organ and there is no obvious melanocortin system in the brain to work on.

Therefore, mice are the least sentient animals that mimic the glucocorticoid induced changes that lead to obesity and diabetes as seen in human patients. As the glucocorticoid effects which lead to obesity and diabetes develop over time, we cannot do these studies in terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The animals in our studies are already closely monitored to ensure they suffer the minimum pain and harm. The small number of animals that undergo surgery get pain medication, and are fed soft food and kept warm after the operation to minimise any pain and suffering. Where animals need injections, the mice will be handled to get them used to this prior to the experiments. We also keep ourselves updated so we are informed of any improvements that we can implement to minimise any harm to the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the PREPARE guidelines as well as any advice we receive from the statisticians, vets and NACWOs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To keep informed of advances in the 3Rs, we regularly check the NC3Rs website. We also receive updates from our animal unit and our NTCO about any changes. We also attend the workshops organised by the NC3Rs and our university to keep informed of new advances in animal welfare and the 3Rs.