Home Office

Animals (Scientific Procedures) Act 1986

Non-technical summaries for project licences granted July – September 2024

Contents

1. Understanding musculoskeletal 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)

Key words

bone, energy metaboilism, musculoskeletal ageing, osteoarthritis, osteoporosis

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 mechanisms underlying musculoskeletal health, and 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?

Musculoskeletal disorders, comprising more than 150 conditions, are at the forefront of age-related conditions affecting approximately 1.71 billion people worldwide. The World Health Organisation has described them as ‘leading contributor to disability worldwide’

giving rise to enormous healthcare expenditures and loss of work. Development, growth, and maintenance of the skeleton are under complex biomechanical, endocrine, and genetic control and depend on integration of cellular events within the skeleton. When this regulation is disrupted, bone and joint diseases including osteoporosis and osteoarthritis, prevail, and this is very common in elderly adults.

Osteoarthritis is one of the most frequent age-related chronic musculoskeletal diseases. The World Health Organisation estimates that 10% of men and 18% of women aged over 60 have symptomatic osteoarthritis. Further, 40% of woman over 50 years are estimated to have an osteoporotic fracture.

Understanding the mechanisms underlying musculoskeletal disorders will provide invaluable insights and will have broad translational potential. It will, therefore, likely lead to long-term patient benefit and societal impact from a contribution to global economic activity. The clinical benefits are potentially huge and their healthcare, financial and societal impact is only set to rise in the ageing population.

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

It is anticipated that the outputs generated from this project will contribute and complement ongoing research into musculoskeletal health. The majority of the UK population will be affected by a musculoskeletal or joint disease as they age. These may include bone loss and fractures characteristic of osteoporosis, osteoarthritis or even cartilage to bone conversion such as in dyschondroplasia. The proposed studies may therefore provide invaluable new knowledge of the mechanisms, specific target molecules, genetic predisposition which lead to these diseases, and ultimately offer new therapeutic approaches. Further, it will generate outputs in the form of: (i) peer reviewed publications (expected >10) (ii) invited seminars (iii) oral and poster presentations at scientific conferences and lay audiences.

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

The proposed studies are anticipated to provide new knowledge of the target mechanisms, molecules, and genes which lead to these musculoskeletal diseases, and ultimately offer novel approaches for treatments. Together, these will benefit immediately the scientific community. The immediate beneficiaries of this work will comprise academic researchers in multiple biological disciplines including, but not limited to bone and cartilage biology, osteoporosis, osteoarthritis, rare bone diseases, and joint imaging. This research will, therefore, benefit a wide range of researchers across the globe, with whom future collaborations can potentially be formed. These partnerships will encourage and facilitate interdisciplinary research and all parties would benefit from this. In the long-term, beyond the 5-year duration of the programme of work, this fundamental research will be extended, refined and may lead to drug targets and development of targeted successful treatments for many musculoskeletal diseases which will ultimately benefit members of the public with these debilitating diseases.

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

I work closely with national and international colleagues where group efforts are made to understand the biological mechanisms underpinning musculoskeletal disease and ageing, and I will continue to nurture these productive collaborations for this work, as well as look to foster new ones where appropriate. I always strive to maximise the outputs of my research and will continue to do so for this work. I am fully committed to ensuring that any

novel results generated are disseminated as broadly as possible across biomedical academic communities and that data are available for future interrogation. Similarly, I will always look to publish unsuccessful approaches as I believe these are just as important in our pursuit of understanding, and within the musculoskeletal field, journals often have special issues focused on these studies. Through the work generated by this project, I will also continue to play an active role in the public understanding of science relating to musculoskeletal, bone and joint diseases, and methods for prevention and management. My research work has featured at various Science Festivals. I have regular community meetings with representatives of elderly adult groups at the largest national charity for elderly people in the UK to increase awareness and understanding on how the research evidence is used to have an impact in society.

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 will use wild type and genetically modified mice and models for achieving the aims of this project. Mice are required to enable us to examine the 'whole joint' rather than the separate components of the joint. We will breed genetically modified mice and use juvenile, adult and aged mice in our experiments. This is because we are interested in understanding musculoskeletal health throughout the life course and, with ageing to improve it across the life course and reduce the likelihood of age- related musculoskeletal diseases.

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

Typically, a mouse will undergo induction of a skeletal pathology such as osteoarthritis either by surgical intervention (destabilisation of medical meniscus) or non-invasive skeletal loading using axial mechanical loading machine to induce post-traumatic secondary osteoarthritis. Before or after this, animals may receive an injection of a compound to examine whether this protects against the skeletal pathology. Mice that will have surgery will receive appropriate levels of analgesia to stop post-surgical pain.

Mice will have food restriction prior to mechanical loading of knee joints and reintroduction of the food. Fasting may be performed on more than one occasion e.g. over a two week period animals will be fasted every second day and knee joint loading performed on fasted or fed animals under anaesthesia. Mice (male and female, young, adult or aged) will receive no more than two regulated procedures, and experiments will last no longer than 8 weeks post-disease induction.

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

The majority of the protocols in this project will not result in any adverse effects. Some mice will have surgery and, therefore, there is a risk of infection, although we will take

every precaution to ensure this does not happen. Our use of surgical approaches will be kept to a minimum and pain relief during our protocols will be achieved through appropriate levels of analgesia to stop post-surgical pain. Axial mechanical loads applied to mouse knee joints will induce strains in the higher range of those experienced physiologically equivalent to strenuous or high-impact activity. Soft tissue damage during application of the loading will be avoided by using polished metal or padded supports as appropriate. There have been no reports to date that describe compromised limb function and lameness in this loading model. Analgesia is not considered necessary after loading. We do not expect to see any adverse effects during fasting of animals. Drugs will be administered at non-toxic dosages and if unknown, this will be carefully tested.

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 anticipated that the highest severity that an experimental mouse will experience in this PPL is moderate such as the ones undergoing surgery. The majority of animals (approximately 60%) will experience a 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?

The aim of this application requires physiological context, and, therefore, this project will largely adopt an *in vivo* approach with mice as experimental model organisms. Use of mice is required to study the systemic changes, so they match as precisely as possible the circumstances in humans. Further, age- related joint deterioration is a 'whole organ' event, and studies that may use models of the joint's constituent components separately would therefore provide insight into only some aspects of the joint function.

Similarly, removing the activity of a gene provides information about what that gene normally does in a physiological (whole body) context and this information cannot be obtained from human studies or cell culture models where, for example, cell-cell interactions and whole body regulatory pathways are lost. Also, the genetic tools and models required for the studies detailed herein are readily available in this species.

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

In vitro ('test-tube') and cell culture-based alternatives have been, and will be, invaluable to biomedical research and I will always consider these in my experimental design wherever possible. I have fully acknowledged their strengths, reviewed their use for others, but I am aware and appreciate their limitations, as detailed below.

Why were they not suitable?

In vitro approaches along with in silico computational models have a number of recognised limitations. Ultimately, they fall short in providing the integrated, organ-level, physiologically intact environment that animal models provide and, thus, make interpretation of indirect effects of agents on bone and other tissues of the musculoskeletal system impossible to detect. These *in vitro* approaches also fail to produce range of structural abnormalities in joint architecture that can be seen, and do not represent all *in vivo* 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?

Numbers of animals are reviewed and based on my previous work. This was used to estimate the minimum number of rodents required for achieving statistically significant differences between groups in each study.

Once we have some preliminary/pilot data, we will look to perform power calculations to determine group sizes for future studies. In-house discussions with a biostatistician will also be held to review our experimental designs.

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 reduce the numbers of animals we use, and routinely use the NC3Rs' Experimental Design Assistant. Power analyses are applied to identify the minimum number of experimental animals needed to answer the scientific question being posed in each study. For example, we have established that in our surgical model of osteoarthritis, a minimum of 6 mice in each group is required to have statistically significant differences. Wherever it is possible we will exploit contra-lateral limbs as internal controls to reduce 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?

Breeding will be performed in a way to maximise efficiency, reduce surplus mice and utilise both sexes in the experiments. Smaller group size pilot studies will always be conducted where appropriate. In our studies, we will optimise the experimental output where possible, thereby, enabling serial data acquisition and removing the need for the humane killing of multiple groups of mice at set time-points. This will be further advantaged by our current attempts to acquire funding for an *in vivo* computed tomography scanner which allows imaging of bone microarchitecture without the need for killing mice.

All experiments using live animals will adhere to the ARRIVE guidelines on design and

reporting. Good principles of experimental design will be applied to ensure sufficient group sizes are used to adequately test the hypotheses. Sample sizes are estimated from pilot studies and previous data using power analysis. Where possible surplus tissues may be made available to 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.

Genetically altered mice bred and maintained are of non-harmful phenotype. Substances are prepared and administered using sterile techniques. The route of administration is via the least invasive method appropriate to the model. The volume of substances to be used, preparing for and undertaking aseptic surgery, and other aseptic techniques will be in accordance with the Laboratory Animal Science Association (LASA) good practice principles and guidelines.

Loading forces will be applied non-invasively using a computer controlled loading device, where loads will be applied to the joints. Drugs will be administered at non-toxic dosages and if unknown, this will be carefully tested. Where surgically approaches are necessary, analgesia will be provided to minimise discomfort following veterinary advice.

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

Here, we will use mice as they are the most suited to be able to answer the biological questions which we pose in each study. The genetic tools and models required for achieving the aims of this project are readily available in this species, the skeletal system of the mouse is similar to human hence it is representative and translatable. Further, mice are required to enable us to examine the 'whole joint' rather than the separate components of the joint, and to take into consideration the whole physiology of the musculoskeletal system. This project will use juvenile, adult and aged mice because we are interested in understanding musculoskeletal health across the life course and in particular, with ageing, and hence more immature life stages cannot be used.

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

The procedures that are in place to administer substances and monitor the animals are 'fluid' whereby any opportunity to refine a technique or ensure additional monitoring is performed where necessary, which is carried out by the licensed scientists. Soft food will be provided to the animals post surgery.

Any relevant refinements made including following surgery are discussed and disseminated to the other users by the animal care scientists. The veterinary surgeon will also offer suggestions for refinements where necessary.

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 ARRIVE (Animal Research: Reporting of In Vivo Experiments). We will also consult the published LASA guidelines on aseptic surgery, guiding principles on good practice and the 3Rs website (www.nc3rs.org.uk).

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

We will maintain close interactions with the relevant welfare, training and information officers. The PPL holder will stay informed of advances in the 3Rs by regularly checking the NC3Rs webpages (https://nc3rs.org.uk/the-3rs) and the newsletters which are circulated monthly. Moreover, the PPL holder will attend appropriate seminars, symposiums and conferences deemed suitable.

2. Bio-synthetic corneal endothelial grafts for transplantation (2)

Project duration

2 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

corneal endothelium, cell therapy, corneal transplant, tissue engineered corneal graft

Animal types	Life stages
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 project is to test the efficacy/safety of our tissue engineered corneal grafts in a rabbit model of corneal endothelial 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?

There is a global shortage of corneal tissue suitable for transplant for patients who have corneal endothelial disease. This leaves many people on a waiting list for a transplant while their vision deteriorates. We and others are developing alternatives to corneal donor tissue to alleviate this issue. We are developing tissue engineered corneal grafts by expanding cells in the laboratory and seeding them onto scaffolds to create biosynthetic tissues. This means that one donor cornea could potentially treat 20-30 patients instead of just 1. We need to test the efficacy and safety of these new tissues so that we can progress the new therapy down the translational pathway to the clinic.

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

We plan to publish the results of these studies in peer reviewed journals and present the findings at national and international conferences. The data will support our patent application that surrounds the synthetic hydrogel. The new data will form part of a new grant application to continue the project.

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

The data from these studies will be published in peer reviewed journals and will benefit our research group- in particular the early career researchers in the group (PDRAs) who will be the first authors on the manuscripts. The data from these studies will lead to new grant applications in the next 1-2 years that will progress the potential therapy along the translational pathway to the clinic. After 5-7 years we expect to have realised the benefit to patients who will receive this novel therapy initially in first in human trials and then further down the line as the therapy is rolled out more widely.

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

The outputs from this work will be published in open access journals allowing it to be disseminated with the biggest reach. The project is a collaborative effort with an international university as well as a cell manufacturing facility and other external partner. All of the project partners will disseminate the results of the work through their relevant channels to increase the reach.

Species and numbers of animals expected to be used

- Rabbits: 57

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 rabbits at around 14-15 weeks. This is the chosen model as the rabbit eye has a similar anatomy to the human eye and at this age, a similar size to the human eye. We require a living animal model because safety and efficacy assessment of the hydrogels requires a complex environment which is not possible to replicate in vitro or ex vivo.

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

The rabbits will be housed in floor pens with space for jumping and ability to rear upwards for scanning and play. Rabbits will be housed socially in groups where possible (females). Males are housed separately but with visual and olfactory contact with others. They have free access to water and standard food and will be provided with additional fruit and vegetables. They have access to tunnels and covered areas and hay for nest building and foraging/play.

During surgical procedures the rabbits will be maintained under general anaesthesia.

Small incisions in the eye will be made (<5mm) to allow access with surgical instruments. The lens of the rabbit will be removed using standard techniques for human cataract removal. One week later a hydrogel graft (with or without cells) will be delivered to the anterior chamber of the eye using a standard technique for lamellar corneal transplant in patients. At the end of the procedure the incisions will be sutured, and antibiotic and anti-inflammatory drops will be given in a tapered regime. Each surgery will last between 30-60 minutes. Each rabbit will receive a maximum of 2 surgical procedures. Rabbits will have their eyes imaged weekly; this is no-contact and requires restraint but no sedation. They may optionally have their blood sampled (weekly). The total experimental period will range from 1 week to 26 weeks.

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

The rabbits will experience some post operative discomfort (inflammation) in the operated eye and will have reduced/no vision in that eye. The contralateral eye will be unaffected. They will receive the same post-operative treatment that a patient would receive after a cataract or corneal transplant- including a tapering regime of antibiotic and anti-inflammatory eye drops (6 a day 1st week down to twice a day after 4 weeks). Any signs of infection will be monitored closely but this is unlikely as all surgery will be carried out under aseptic technique which at least meets the standards set out in the Home Office (HO) Minimum Standards for Aseptic Surgery. During the periods of restraint for imaging or blood sampling the animals may experience some distress and some modest discomfort from the needle stick. The methods of restraint are standard to the handling of rabbits and are nearly always well tolerated by the animal. The technical staff handling the rabbits will be experienced and also familiar to the animals who will have spent considerable time with them in the initial acclimatisation 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)?

The expected severity is moderate 100% of rabbits.

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?

Safety and efficacy testing of the peptide hydrogels requires their assessment in a complex environment that is similar to the planned location for the therapy in patients. It is not possible to completely replace the use of animals in this study as we require a physiologically relevant model to test our bio-synthetic graft before moving to clinical trials in humans, the next step in the translational pipeline.

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

We conducted initial experiments using in vitro systems (human cells on scaffolds in culture) and we used human eye tissue to conduct ex vivo experiments to optimise the hydrogel delivery techniques.

Why were they not suitable?

We require a complex environment with whole body systems (including immune responses) to study safety in the eye and this can only be achieved with in vivo 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?

We have conducted pilot experiments to estimate numbers of animals required in each group and consulted the published literature on similar studies assessing other tissue engineered solutions as well as ISO standards on safety testing.

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

Initial experiments used porcine/rabbit ex vivo models to refine our transplantation and cell culture techniques. We used shared eye tissue from other researchers who did not need the tissue and we also used human tissue in ex vivo models. This allowed us to limit the number of experimental groups, and so animal numbers, that we require for the in vivo experiments.

Statistical design has been conducted with the help of a statistician to determine the lowest number of animals that will be required to give statistical significance. Power calculations were based on pilot studies and similar published studies.

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 tissue with any other users that require tissues that we do not require. We only ever order the number of animals we require for an experiment from an external breeder. We have undertaken pilot studies to determine numbers required for larger 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 have chosen the rabbit as our model due to its similarity to the human cornea in terms of corneal layers and dimensions. Eyes of laboratory rodents are too small for the highly specialised surgical instrumentation required for this procedure. Consequently, use of rodents would limit the ability to achieve the scientific aims of the project. Rabbits are the lowest species able to provide the globe size and structure for this work.

The endothelial damage caused by removal of the endothelial layer (and associated Descemet's membrane) is a gold standard model of endothelial damage, as endothelial cell loss is the cause of the clinical problem in patients so it mimics the disease state accurately.

This model involves eye surgery as would be performed on human patients, so surgical techniques have already been refined to reduce pain and suffering.

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

Eyes of laboratory rodents are too small for the highly specialised surgical instrumentation required for this procedure. Consequently, use of rodents would limit the ability to achieve the scientific aims of the project. Rabbits are the lowest species able to provide the globe size and structure for this work.

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

To reduce animal distress, we will be operating in only one eye so rabbits will have good vision in one eye at all times. Post-operative analgesia (as advised by the NVS) and antibiotic/anti-inflammatory treatment will be administered to minimise discomfort and risk of infection. Animals will be monitored closely for signs of pain and distress (rabbit grimace scale). If this cannot be relieved by medical treatment then the animals will be killed by schedule 1 methods. It is possible that surgery may result in post-operative infection or significant tissue inflammation; in the unlikely event that this occurs, the animals will be killed for welfare reasons.

All surgical procedures have been optimized using ex vivo rabbit eyes and cadaveric rabbits prior to in vivo surgery, which reduces the risk of complications occurring in the in vivo animals. All surgery will be performed by a consultant ophthalmologist who is highly trained in the transplantation procedure, performing similar procedures on a weekly basis in patients. This means that the rabbits will receive similar care and attention that a patient would expect. The surgeon has now also had considerable experience operating on rabbits (50 operations) with no complications.

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

We follow the NC3Rs published guidance on, for example, housing and husbandry of rabbits. Where we can, we house rabbits in social groups rather than alone, as suggested

by this guidance. (https://www.nc3rs.org.uk/3rs-resources/housing-and-husbandry-rabbit)

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

I subscribe to the NC3Rs newsletter which updates readers with developments in the 3Rs area and give examples of best practice. Through our animal unit we also receive info on best practice. As new information is made available, we aim to modify our studies to incorporate best practice e.g related to housing and husbandry or rabbits.

3. Interactions between metabolism and epigenetic control in ageing health

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

metabolism, epigenetics, 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?

How genes are organised and regulated in cells can depend upon metabolites – small molecules present in our cells – that come from our diets. We want to study how these connections change during the lifetime and affect resilience of tissues, and whether interventions can be made to improve metabolic and epigenetic health in 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?

Our genes are wrapped by proteins to form chromatin which helps condense the DNA in the cell nucleus and enables genes to be controlled properly during development and in different tissues. These normal processes are often referred to as epigenetic control of genes. Metabolites – small molecules present in our cells that ultimately come from our diet – are needed to allow different chromatin states to be adopted - influencing the availability of genes for expression. This is important as stem cells differentiate to create defined cell types. But we do not understand how the availability of the metabolites influencing chromatin processes is controlled, as the same metabolites are also used in

many other processes in cells. We believe linkages between metabolites and chromatin change as we age, or in response to poor nutrition or food excess, and may contribute to how tissues lose proper function with age. We should like to test whether we can control metabolite availability to benefit ageing – we know we can do this in simple organisms, such as yeast, using cellular pathways that also exist in mammals. This could provide a rationale for diet interventions that could promote ageing health.

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

The key outputs will be new understanding of how chromatin states critical in regulating gene expression are controlled by metabolites, and how this balance changes over the life-course, with impacts on tissue function, metabolic and ageing health. These findings will translate firstly into peer- reviewed publications, but they will also inform public engagement and dialogue work we plan to undertake. In addition, beneficial metabolic interventions we discover may be protected as intellectual property.

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

(i) The academic scientific community, particularly in relation to epigenetics and metabolism. Our research will provide new, fundamental understanding of the interface between cellular metabolites and chromatin mechanisms, and how these linkages change with age. Our outputs will also contribute to future studies: new datasets and new genetically-altered (GA) strains will be generated and made available to other users to advance future research.

(ii) Funders, in particular the BBSRC. Our research underpins the delivery of a strategic priority of the funder "*Bioscience for an integrated understanding of health*", including "*to enable new mechanistic understanding of key biological mechanisms underpinning health including the biological basis of ageing*".

(iii) Clinicians working on metabolic disorders. Our outputs will help provide scientific rationale for the influences of diet and lifestyle on ageing health.

(iv) Policy makers and the general public. Benefits will include increased knowledge, understanding and awareness of the importance of epigenetics and metabolism, including the impact of diet on long- term health, and its potential social and economic relevance.

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

We have many mechanisms in place to do this in addition to the immediate academic routes of presentations at international conferences, publishing research papers in open-access journals and making datasets, new reagents and GA mouse strains openly available. The Institute has an expertly- qualified Knowledge Exchange and Commercialisation team, which has an excellent record in supporting Institute researchers in finding commercial partnerships to translate findings of potential translational significance. The Institute also has dedicated Public Engagement and Communications teams, which have an active programme of public engagement events and contribution to policy initiatives, in particular to provide expert advice around potential interventions targeting ageing. And we expect to build on and develop new academic collaborations based on our capabilities and new knowledge.

Species and numbers of animals expected to be used

- Mice: 12,750

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 the project are to understand the linkages between metabolites and chromatin mechanisms, and how these change in ageing to result in impaired metabolic health relevant to human health. It is necessary to undertake this in an animal model, because cell models do not adequately recapitulate changes that occur in ageing. We have chosen mouse as our animal model, because it possesses many metabolic characteristics relevant to chromatin regulation similar to humans and, for a mammalian species, has an ageing lifespan that can be studied within practical timescales. In addition, we are able to benefit from existing genetically altered (GA) strains for refinements such as tissue-specific control of the genetic modifications we may introduce.

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

The most common procedure in this project is breeding and maintenance to produce adult mice that will be killed via approved Schedule 1 to supply cells and tissue for the aims described in this project. Some mice will be kept until they are aged to ~28-months of age. Breeding will include genetically altered (GA) mouse strains, and we shall also be generating new GA strains, including the use of highly-refined genetic modifications that will selectively affect genes in tissues of interest, such as fat cells or the liver.

Small numbers of mice will be fed altered diets, such as high-fat diets or diets with reduced amino acids, to evaluate the effect of diets on how the genes we are interested in are controlled. These diets are not expected to cause distress but may result in obesity, or minor weight loss. Some mice will have blood sampled to measure glucose and other metabolites; these mice are not expected to experience more than mild and transient discomfort from blood sampling.

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

Many of the genetic alterations in mice we breed are not expected to change function of genes (for example, they are tags to help us isolate cells of particular interest to high purity); breeding of these strains is not expected to have adverse effects.

For genetic alterations that we suspect could cause a harmful effect if present in the whole animal, we shall use genetic methods to limit the alteration to tissues of interest (e.g., fat cells, liver) thereby minimising the risk of adverse effects.

Some genetic alterations may alter metabolism in mice, leading to possible longer-term conditions such as obesity or diabetes; these conditions will be monitored to avoid development of harmful outcomes.

Mice fed altered diets, e.g., high-fat diet over a period of two to three months are expected to become mildly obese and diabetic, but these will be monitored to avoid development of

harmful side-effects.

For mice that undergo surgery, mostly for transferring embryos, the duration of anaesthesia and surgery is very short and the animals are expected to make a full and unremarkable recovery, although analgesia will be administered to mitigate short-lived pain.

Mice being administered tracer or labelling substances, which may be done by injection or orally, are not anticipated to experience any adverse effects, beyond transient discomfort due to the injections.

Mice administered insulin or substances that mimic adrenalin action may experience short-term reduction in blood glucose which, in rare cases, could cause altered behaviour or fitting. Mice would be continually monitored and if such effects occur they would be treated by injecting glucose to restore normal blood sugar levels.

For mice that are allowed to reach advanced age (>18 months), we do not anticipate specific impacts or adverse effects in mice ageing healthily. Animals that begin to show irreversible ill health, such as sustained weight loss, will be killed because we are interested in healthy ageing and not the ageing of diseased or unhealthy 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)?

The expected overall severity of this project licence is Mild, with fewer than 10% of animals expected to experience a maximum severity of Moderate.

Procedures involved in the generation of new genetically altered mouse strains: 50% moderate; 50% mild

Breeding/maintenance genetically altered mouse strains: 5% moderate; 10% mild; 85% sub-threshold

Metabolic and diet studies in mice: 5% moderate; 95% mild

Ageing of mouse strains: 5% moderate; 95% mild

Administration of antibiotics to mice: 5% moderate; 95% mild

Administration of chromatin-modifying agents to mice: 5% moderate; 95% mild

Administration of β3-adrenergic inhibitors to mice: 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?

We are seeking to understand how the availability of metabolites critical for chromatin regulation, and thus for fidelity of cell state and function, changes with age and may contribute to loss of tissue resilience that accompanies ageing. We are also planning to identify interventions that could mitigate these age-related declines. We need to conduct some of this work in whole animals, because ageing cannot adequately be modelled in cell-based systems.

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

We are able to perform parts of our work in *in vitro* mammalian cell systems – those that investigate how our chosen genetic modifications alter metabolic pathways and chromatin processes outside of the context of ageing. These *in vitro* systems help refine the experimental approaches before applying them to mouse models.

Why were they not suitable?

The main limitations of mammalian cell culture systems are that they cannot properly recapitulate the process of ageing, nor faithfully reflect responses to interventions such as altered diet. In addition, metabolic interventions within specific cells could have effects systematically or on other organ systems, and these potentially beneficial or adverse effects would be missed in cultured cells that represent single specialised cell types.

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?

From experience of similar experimental designs in previous projects. With advice from the Institute statistician in relation to the minimum number of animals (data points) necessary to achieve statistically robust results in any procedure with a quantifiable outcome. Annual Return of Procedures data from our current licence to estimate the number of animals that we will need to use for breeding.

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

For the design of quantitative studies we generally follow the ARRIVE guidelines and can use power analysis to determine sample sizes. We typically use a significance level of 5% and a power of 80%, estimating standard deviation from pilot experiments if necessary or from comparable prior studies. We include advice from the Institute statistician in addition to making use of online tools, such as the NC3Rs' Experimental Design Assistant.

We can reduce animal numbers needed by making multiple measures from the same animal or sample, wherever possible. For example, current protocols for molecular profiling of tissues *ex vivo* enable us to obtain measures of gene expression and chromatin state in the same assay; metabolomic analyses can profile >90 metabolite species in a single sample.

As well as reducing the total number of samples, thus animals, needed to obtain these measures, obtaining multiple data from the same sample represents a refinement in 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?

Use of power calculations for optimized animal group sizes based on comparable data from previous experiments and advice from the Institute statistician.

Minimising inter-group variability using controls of matching age, sex and genetic background.

Cryopreservation of strains when no longer required.

Use of colony management software that helps avoid overproduction.

Collection and freezing of tissues post-mortem beyond those needed for our own experiments, to make available to other researchers who may be interested in similar questions (e.g., aged tissues).

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 mouse as our animal model, because it possesses many metabolic characteristics relevant to chromatin regulation similar to humans and, for a mammalian species, has an ageing lifespan that can be studied within practical timescales.

Some mice will be allowed to grow old (>18 months), because we are studying how linkages between metabolism and chromatin regulation change with age: animal house staff are trained and experienced in the recognition of signs of ill health that could be found in ageing animals and we would expect to humanely kill animals that show signs of suffering that is greater than minor and transient or in any way compromises normal behaviour.

Some mice will be fed altered diets and/or challenged metabolically, for example, by injection of glucose or adrenalin mimics: this will be done to test how new genetic

modifications of our genes of interest alter metabolic pathways or lead to changes in function of metabolically relevant tissues.

Some mice will be administered substances by injection or oral gavage for metabolic tracing to test how they are incorporated into chromatin; the substances and routes of administration are not expected to cause anything other than transient discomfort.

Some mice will undergo surgery for implanting embryos to generate new genetically altered strains. The duration of anaesthesia and surgery is very short and not expected to cause more than short-lived pain, which can be controlled with analgesia. Non-surgical embryo transfer methods will be used where the success rate matches that of surgical embryo transfer methods or is sufficient for the aims of the experiment.

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

The focus of our study is how metabolic and chromatin integrity changes during the life-course and into the aged mammal, so we need to maintain mice over a range of adult stages. In addition, we are studying the linkages between metabolites and chromatin mechanisms particularly in tissues (fat) that are metabolically relevant in warm-blooded mammals rather than cold-blooded vertebrates or less sentient animals that are physiologically less similar to human physiology.

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

Harm to animals is minimised by using sterile conditions, anaesthetics in surgical procedures; humane methods of killing; regular surveillance to quickly identify deviation from health; and by targeting possibly harmful genetic mutations to the cells of interest (e.g., liver, fat tissues) to avoid the possibility of whole-animal suffering.

Housing, husbandry and care conditions are provided by a dedicated Biological Support Unit (BSU), staffed by highly-trained animal technicians and overseen by experienced supervisors and NACWOs. The BSU enjoys permanent and expert veterinary cover.

If, in rare circumstances, an animal has an unexpectedly severe response to a procedure or diet, or where an infection develops, treatment is given where possible and, if necessary, the animal is humanely killed.

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

Planning and conduct of the project is informed by the ARRIVE and PREPARE guidelines.

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

We keep fully aware of developments in cell-based and organoid systems that could REPLACE some of the animal use and which could be of benefit to the aims of the project through the published literature, conference attendance and interactions with science groups working on related questions, and would adopt them where we can, if they prove reproducible and representative of the *in vivo* situation. We also remain in regular communication with the Institute's animal facility staff about husbandry and procedural developments that could provide gains in REDUCTION and REFINEMENT. The animal

unit maintains an up-to-date online library of 3Rs resources.

4. Mechanisms of Normal and Leukemic Haematopoiesis

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

Hematopoietic, Leukemia, Ageing, Haematopoiesis, Stem Cell

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

(i) To gain novel insights into the function of haematopoietic stem cells (HSCs), which are responsible for blood production throughout life, and to understand how HSC function changes with age. (ii) We explore how acquired genetic changes corrupt HSC function to cause Acute Lymphoblastic Leukaemia in all age groups, and how age-related changes in normal HSCs increase the risk of chronic inflammation, anaemia and the incidence of myeloid cancers in older people.

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 treatment for lymphoblastic leukaemia is long and arduous for patients of all ages. Although outcomes in children have improved, treatment can cause lifelong complications. The disease is much harder to treat in adults and is incurable in the over 60s. More effective and less toxic treatments are required, and this is a key aim of our programme (ii)

Age related changes in the production of white (myeloid) blood cells underpin many chronic inflammatory and degenerative disorders in older people and are also responsible for the higher incidence of anaemia and cancers of white blood cells with age. However, there is little understanding of how these changes come about and how they can be mitigated.

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

1) Improved understanding of the regulatory processes operating in normal HSCs, and of how dysregulation of these processes leads to leukaemia.

2) Improved understanding of how, and in which cells, the genetic mutations that cause leukaemia first arise

3) The identification of new therapeutic targets for the treatment of leukaemia
4) Further characterisation of those leukaemic cells which resist chemotherapy, and which ultimately cause relapse

5) Understanding of the cellular basis of ageing in the hematopoietic system and its clinical impact

6) Advancement of knowledge in the field. Outputs will be in the form of publications in peer reviewed scientific journals, and presentation of data at national and international scientific meetings.

7) Over the longer term, identification of new diagnostic biomarkers, new therapeutic targets and/or modes of treatment.

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

In the short and long term, the scientific community (including academic scientists and translational physicians) will benefit from our publications and presentations at national and international scientific meetings. In addition, we have participated in regular public outreach programs for many years, where we have explained the benefits of our research to members of the public during various events at our establishment organized in collaboration with the cancer charities and philanthropists who fund our work. we intend to continue to participate in these types of public engagement activities.

Intermediate term: The identification of new markers (genes or proteins) that can be used to identify and/or purify cancer cells would be of benefit both to scientists studying leukaemia and - in the longer term - to patients.

Longer term: identification of new therapeutic target and/or /modes of treatment would benefit patients. The population at large would also benefit from an increased understanding of the first DNA mutations that happen in leukaemia, and the type of cells in which they arise. Cells which carry these first mutations are usually non-cancerous, but carry an increased risk of subsequent events making them cancerous. In blood cancer, this is called "pre-leukaemia". A better understanding of pre-leukaemia may open the way for large scale screening of the healthy population with the aim of identifying those at increased risk of leukaemia, and possibly offering them safe and targeted therapies to eliminate “pre-leukaemic” cells or, at least, prevent their progression to leukaemia

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

Publications in peer reviewed scientific journals, and presentation of data at national and international scientific meetings.

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 only animals to be used in this project will be mice. The haematopoietic system in the mouse has been extensively studied over the last 60 years and mouse HSCs have been used as a paradigm for understanding human HSCs functionally. Although both human and mouse HSCs can survive in tissue culture conditions, they rapidly lose their functional properties when they are outside their natural environment (I.e., the bone marrow). Currently the only way of studying their function experimentally is to do so when they are resident in living bone marrow , or shortly after harvesting them from the bone marrow.

One aspect of this project is to study the changes that occur in the haematopoietic system with age, including reduced fitness and increased myeloid bias of the hematopoietic stem cell (HSC) compartment, causing increased risk of immune compromise, anemia, and malignancy in humans. Studies of ageing are only relevant in the context of a whole organism and cannot be performed using cell line models. Aged mice will be used as they display similar changes in their blood system to aged humans. Bone marrow will be harvested from aged mice (> 18 months) and the cells assessed by a range of molecular and functional assays to understand how the cells change with age. The impact of regimes proposed to reduce, delay or reverse ageing will be assessed by performing the same experiments using bone marrow harvested from mice treated with these regimes. Other tissues may be harvested at the same time as bone marrow if appropriate. The effect of age on the bone marrow microenvironment may also be explored, including by transplantation of cells into aged mice.

Mice will be used at various life stages, appropriate to the question being addressed.

Adult : Adult mice will be used both as a source of normal cells and as recipients of transplanted cells (normal and leukaemogenic). They will also serve as controls in some experiments to study the effects of ageing.

Aged : Aged mice will be used to study the effects of ageing on the haematopoietic system.

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

In the case of the creation, breeding and maintenance of genetically altered mice, mice will either be sought from commercial suppliers or created ourselves using standard well-established procedures.

Therapeutic substances will typically be administered in the food or water for the entire life of the mice but may also be administered daily for shorter periods, for up to a few months, through routes such as oral gavage, injection or in the food or drink.

During ageing studies, mice will be kept up to a maximum of 30 months, subject to satisfactory results of health monitoring. Mice will be culled earlier where consistent with the experimental plan or if required for humane reasons - for example, they reach maximum acceptable tumour burden or the limit of acceptable deterioration of health through observable body scoring. Measurements of blood parameters may be done throughout the lifespan of a mouse.

Mice will also undergo injections of cancer cells, and prior to this some mice may be given a dosage of radiation to enable the acceptance of the cancer cells administered. These studies last for around 2 to 6 months and allow us to understand the growth and how the cancer reacts to treatment regimens that are also used in humans.

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

Giving mice leukemia: 100% of animals will experience weight loss after irradiation over a period of 1 to 10 days and not more than 20%. Development of leukemia may lead to for example, staring coat, labored breathing and hunched posture depending on how aggressive the leukemia is. On experience this happens in less than 10% of mice due to careful monitoring, and past experience with disease progression.

Chemotherapy of mice with leukemia: Due to the toxic nature of the chemotherapy drugs we use (which are the same used to treat children) animals will experience weight loss, staring coat, hunched posture, after 7 to 10 days of treatment. This persists until the end of the dosing schedule which lasts 4 weeks all animals are monitored daily during this time to ensure their welfare.

Ageing in mice: Animals that are allowed to age to the full 30 months in this project may develop conditions associated with ageing which are the same as humans like deterioration in overall general health. However, the majority of animals do not progress this far and are sampled at an earlier timepoint.

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 20% of all animals on this project will be used in creation, breeding, and maintenance of genetically altered mice should cause no more than mild severity 100%.

Approximately 30% of animals will take part in ageing studies, those mice will mostly experience sub- clinical signs and will mostly be mild severity. A small subset of animals (approximately 3% of animals on this project) will be allowed to age to the full timeline of 30 months some of these may become moderate.

Where we have injected cancer cells into the mice without treatment (approximately 7% of animals on this project) will only reach a mild severity before the humane endpoint. In cases of mice undergoing treatment, (approximately 40% of all the mice) will experience a

moderate severity due to the treatment before reaching a humane endpoint.

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?

Mice are used in this study because they resemble humans in many ways - anatomically, physiologically, and genetically. All mouse work is preceded by intensive studies done in the laboratory. However, while in vitro studies can provide important molecular and cell physiological insights they cannot capture the complexities of either normal or pathological physiology.

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

For our leukaemia studies, we considered the in vitro use of established human leukaemic cell lines, and also in vitro culture of primary patient leukaemia samples. We also considered using human induced pluripotent cells, genetically altered to express mutations associated with leukaemia.

Why were they not suitable?

Established human leukaemic cell lines were not suitable as an alternative to patient samplesin this project as they do not adequately represent the complexity of human leukaemia cells as seen in patients.

In vitro culture of primary patient leukaemia samples was not suitable as an alternative to xenografting into mice as this is rarely successful, leading to the loss of valuable clinical material with no scientific benefit. In addition, in vitro culture does not reproduce effects of the in vivo bone marrow microenvironment.

Induced pluripotent stem cells (IPSCs) are a type of cell that has the potential to produce all different types of human cells and can be genetically modified in the laboratory with high efficiency. Genetically altered IPSCs are valuable for studying the specific effects on haematopoietic cells of a single genetic alteration associated with leukaemia, or combinations of two or even three such alterations. But IPSCs do not generate full leukaemia and therefore cannot be used to model the complexity of genetic alterations that co-exist within a single leukaemic patient sample. Furthermore, the development of B cells in vitro from IPSCs is inefficient, limiting the downstream analyses that can be performed. IPSCs are therefore useful for addressing specific questions about the mechanisms at work in leukaemia, but discoveries made in IPSCs need to be confirmed in genuine leukaemic cells.

Culturing normal human HSCs (from cord blood or bone marrow) in vitro was not suitable as these cells do not maintain their stem cell characteristics well in vitro, making

experimental reproducibility a challenge. After a period of in vitro culture, it is necessary to test their stem cell potential, for which the gold standard assay is transplantation into immunodeficient mice. In addition, xenografted human HSCs are in an environment more relevant to the normal situation than HSCs in culture.

In essence, there are no plausible non-animal alternatives to study HSC biology – we cannot replicate either normal HSC function or human leukemogenesis 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 numbers of mice we would like to use for the experiments would primarily have been based on previous experiments or those performed in the literature or by collaborators. We have also looked at statistical analysis for numbers of animals in experiments. In this way we are confident to produce meaningful scientific data with the least number of mice possible.

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

Mouse colonies will be closely monitored to avoid excessive breeding. Further reductions in the number of mice will be achieved by improved study design and statistical methods which will allow us to integrate and aggregate the data across multiple experiments thus achieving a statistical conclusion which makes additional experiments with animals superfluous.

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 plan our experiments in advance and breed/maintain the minimum number of mice required. Our earlier experience demonstrates our understanding of the numbers of mice required to generate statistically robust data in normal and leukaemic HSC assays. We will follow best guidance practice published on websites such as NC3Rs and PREPARE 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.

Mice are chosen because they are the most frequently used mammalian model system to study biology of stem cells and cancer. Other advantages of the mouse model include the availability of antibodies for the identification and purification of different classes of haematopoietic stem cells and mature blood cells, and the availability of a range of tests of stem cell and leukaemic cell function, such as their capacity for cell division and their ability to survive transplantation into recipient animals and to subsequently regenerate normal blood or blood cancers. Previous work from our group and others showed that immunodeficient mice tolerate transplantation of both normal human blood stem cells and leukaemic cells. Normal human blood stem cells then divide and mature to generate most cells of the human blood system within the mouse environment. Transplantation of leukaemic cells obtained from patients into mice gives rise to leukaemia, which is first established and then maintained by a subset of the cells called leukaemia-propagating cells. In this system, the cellular complexity of a leukaemic sample – in terms of genetic and functional differences between cells in the same patient sample - is reproduced after transplantation.

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

Our overall aim is to understand normal and leukaemic adult human haematopoiesis, and the impact of normal ageing on haematopoiesis. The mouse is the only animal model in which adult haematopoiesis has been well-characterised to date, and has been found to closely mirror that of human. This similarity extends to the effects of many of the gene mutations identified in leukaemia, and the changes seen in ageing. In addition, immunodeficient mice are the only model that has been developed that allows the transplantation of human cells, so that their function can be studied in an in vivo setting. Less sentient species such as zebrafish and frogs have been used for some studies of haematopoiesis, but their greater evolutionary distance from humans makes them less relevant than mice, and they are not amenable to human cell transplantation. When we transplant leukaemic cells into mice, it is important that the mice are large enough to allow sampling and analysis of their bone marrow once the leukaemia has developed, which may in some cases be just a few weeks later. Neonates could in principle be used for leukaemias that are already known to be slow to develop, but these are unusual, and it would not be scientifically rigorous to use recipients of very different ages for different leukaemic samples. Neonates are therefore not suitable as recipients in this project.

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

All our procedures are designed to minimise pain to the animal as much as possible. Yet, certain procedures will have a moderate impact on the wellbeing of the animal. Thus, we have developed predefined endpoints to prevent and avoid the onset of any adverse effects and to keep the negative impact on their welfare as low as possible. These pre-defined endpoints take different parameters into account such as behavioral changes, body weight etc

Also, during any study mice are monitored closely to ensure mice do not experience excessive adverse effects. The researchers are experienced in the studies undertaken and work closely with the animal care staff to keep them up to date on studies that are ongoing.

In addition, the institute in which the animal procedures are carried out runs a comprehensive health monitoring programme. Animal health and welfare records are

maintained to include any adverse effects that may develop, particularly in genetically altered and spontaneous mutant strains. Signs consistently associated with a particular phenotype/genotype will be recorded on the respective “information sheet” in the breeding area and the mice will be maintained under conditions where their health status can be protected as far as it is reasonably practicable.

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

We will follow the best guidance practice published on websites such as NC3Rs and PREPARE guidelines and workman et al.

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

By literature searches and staying up to date with the NC3Rs website and attending NC3Rs symposiums. Checking with the NVS, NACWO and animal care staff before any procedure is carried out so that we can implement these techniques effectively. Collaborating with researchers who have carried out this work before.

5. Neural circuits linking appetite, reward and cognition

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

motivation, feeding behaviour, cognition, neural circuits, addictions

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

Cognition has evolved to, among other things, meet the metabolic demands of an organism. This research aims to understand how specific cognitive and metabolic brain regions interact for guiding appropriate food-seeking 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?

A fundamental understanding on the brain circuits involved in guiding our decisions related to what and when to eat can provide important insight on not only the neural mechanisms

disrupted in eating disorders but also how motivational process are dysregulated in various forms of addiction.

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

The primary expected outputs of this work are new knowledge about the following areas:

How the brain and behaviour is changed by consumption of different nutrients (e.g. protein, amino acids, or other macronutrients) or deficiency of a specific nutrient. In particular, what brain circuits are activated and how is learning affected?

What are the behavioural strategies that animals use to search for and obtain food. Specifically, how decisions are made regarding food choices and how food-seeking actions are coordinated.

How consumption of different nutrients or nutrient deficiency affects responses to particular foods (e.g. obesogenic diets) and other rewards (e.g. drugs, social interaction).

How the brain learns about food and other rewards and how the brain makes choices between feeding and other behaviours.

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

Knowledge resulting from each of these items will be primarily published as peer-reviewed articles. In addition, preliminary findings will be presented locally and at international conferences. This strategy will result in knowledge being used by a number of interested parties. The predominant beneficiaries will be the academic community who study the neurobiology of feeding, the neuroscience of reward, decision-making and motor control. They will use our findings to inform their research, generate new hypotheses, and refine their protocols. Clinicians and other healthcare professionals will also benefit from this increased knowledge and will be able to extrapolate from this animal research to devise new studies and translational hypotheses. Finally, the general public and lay audience may benefit from increased knowledge about why we choose certain foods and ways in which we might combat obesity and addiction via behavioural change .

In addition, presenting at conferences allow for rapid communication to the community and findings specifically related to animal welfare can be quickly disseminated via our local NC3Rs network. Beyond the lifetime of the project, there may be translational gains to be made, for example, understanding better how humans regulate appetite and how responses to food influence other aspects of human behaviour, such as mood or addiction. For example, if we understand the biological processes such as the neurotransmitters, hormones or receptors that underlie a particular aspect of feeding such as binge eating, we might be able to target them pharmaceutically, an approach we are undertaking with collaborations across different academic institutions. In addition, knowing that particular constituents of our food (e.g. sugar, fat, or even artificial sweeteners) have effects on eating habits will allow us to shape public policy and education in a way that makes people healthier. Our research also aims to understand disorders affecting metabolism, motivation and motor control such as Alzheimer's and Parkinson's Disease and so we plan on disseminate our findings and in general knowledge regarding these processes to the general public, specifically those suffering from such disorders and their family members/caretakers.

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

In order to maximise the output for this work we will look for any opportunity for making the outcomes available to the scientific community and to the general public. Our group has a good track record at publishing our findings and we see no reason that this will not continue. When publishing our work we will work with ort press office to make our findings public within the university as well as throughout social media platforms. We will always highlight the societal impact in our description so that clear connections can be made between the fundamental science and the potential impact on society. This will help in communicating the importance of performing thus type of research responsibly for the good of society as a whole. In addition, the work will be communicated at professional conferences but also at open days and at science festivals for communicating science to the public.

It is common for our research to be collaborative and we will make sure that a similar press release will go out in all affiliated institutes for the purpose of not only disseminating the new knowledge but also make known the potential for new collaborations locally and internationally.

When designing an experiment it is the case that some approaches are unsuccessful We see no reason why these should not be included in the published work and will make a point to do so in all future studies.

Species and numbers of animals expected to be used

- Mice: 700
- 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.

We plan on using adolescent and adult rat and mouse lines for related neural circuits with behaviour. To do so we will use rat and mouse lines that show cell-type-specific expression of enzymes for the purpose of identifying neurons, monitoring and manipulating cell activity. These techniques allow for 1) identifying the location of relevant cell types 2) relating their activity to specific behaviour states and actions 3) determining causal relationships between activity from specific cell types and behaviour.

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

In order to establish a causal relationship between neural circuits, feeding behaviour and cognition we will need to express various proteins used for imaging and manipulating activity in and ex vivo. To do so we plan to (Protocol 1) breed transgenic mice and rats strains that allow us to genetically insert appropriate experimental proteins for imaging and manipulating specific cell types as well as (Protocol 2) have mice and rats engage in behavioural paradigms used to assess behaviour towards rewards such as food and how cognition can support effective foraging in a controlled experimental setting. For this mice and rats will at times be put on a restricted feeding schedule and weight will be measured regularly. These experiments can last for up to one year. In Protocol 3, tracing dyes and

other substances (i.e. viral constructs) for determining the functionality of specific neural areas/circuits will be intracranially injected when animals are either at an adolescent or young adult stage of development.In some circumstances we will permanently implant a cannula intracranially for infusing drugs/ substances before and during an experiment or a device for measuring neural activity (i.e. electrode, fibre optic cable) in behaving animals. Similar to Protocol 2, these experiments can last for up to one year in duration.

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

1. Animals may be food-restricted and so there is the potential for suffering through weight loss. However, weights will be monitored frequently and if animals drop below 90% of free-feeding controls, measures will be taken (additional feeding) to allow them to gain weight back.

2. Surgical procedures have a risk of pain or discomfort following surgery. This will be minimized by appropriate dosing of analgesics (e.g. given peri-operatively, e.g. before, during and/or after) and aseptic surgical procedure to reduce risk of infection. In rare instances (<5%), intracranial surgery may result in adverse effects such as impaired movement or seizures upon waking from anaesthesia. Animals will be closely monitored after surgery, especially in the first 72 h, during which they will be checked at least twice a day and humanly euthanised if there is any indication of the animal suffering and/or not recovering well from anaesthesia.

3. Experiments involving expression of non-harmful genetic modifications (i.e. via dosing of substances) that are not expected to lead to adverse effects but there will be careful monitoring for possible side effects.

4. During experiments using pharmacological manipulation, as stated above, only compounds in which temporary adverse effects are known will be minimally used.

5. In experiments with intracranial implantations, head-caps may occasionally (<10%) become loose or detached either in the home cage or during recording sessions. Animals will be regularly monitored and in these cases, animals will be humanely euthanised.

6. During terminal procedures at the end of experiment, animals will be deeply anaesthetized throughout and so adverse effects are not expected.

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 breeding and behavioural Protocols (1&2) the expected severities are mild while for Protocol 3 where surgeries will be performed we expect the severities to be moderate. Transgenic mice bred under Protocol 1 will go onto procedures with mild (Protocl 2: approximately 35%) and moderate (Protocol 3: approximately 65%) severity. Transgenic rats bred under Protocol 1 will go onto procedures with mild (Protocol 2: approximaely 40%) and moderate (Protocol 3: approximately 60%) severity.

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?

We need to use animals - in this case rats and mice - to understand how the brain is involved in feeding and how cognition can support effective foraging behaviour. To measure what is going on in the brain, we will need to manipulate specific neurons and insert devices which cannot be done in humans. Also, it is impossible to work out how the stomach and other organs talk to the brain without studying it in a whole animal.

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

We considered using a novel experimental preparation called the organoid - a 3D culture of neuronal aggregates which can be beneficial in determining the physiological changes associated with drug delivery in addition to abnormal changes associated with certain neurological diseases. In addition, we are considering complementary work in humans using non-invasive brain imaging techniques to monitor activity related to food-seeking behaviour in both healthy individuals and eating-disorder patients.

Why were they not suitable?

For this purpose of our proposal the organoid preparation is largely unsatisfactory since our goal is to better understand the circuits involved in complex decisions and behavioural manifestations associated with feeding behaviour and this prep would only allow us to measure cellular responses to applied drugs or inserted or deleted genes.

There are a number of limitations for non-invasive imaging in humans. 1) Low spatial resolution for identifying the exact regions and cell types related to behaviour, 2) inadequate temporal resolution in relating brain activity to behaviour and 3) unable to precisely determine the causal mechanisms between brain circuits and 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 have estimated these numbers based on our prior work with research rodents. These numbers are based on breeding, colony maintenance, ratio of generated hetero/homozygote offspring, experimental piloting for full anatomical and behavioural experiments.

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 so that they use the smallest number of animals possible. One way of doing this is by using maths to work out exactly how many should be needed to see an effect. Also, by looking at different measurements in the same animal, instead of using more animals for different behavioural paradigms, we can reduce the numbers that we need. When appropriate we will use minimally invasive behavioural and imaging techniques (i.e. time-lapse imaging in within animals instead of between animals) for reducing animal use totals.

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 way we have of reducing numbers of animals is to design paradigms that use repeated testing so that within-subjects statistical analysis can be used. As such, our recent adoption of photometry, which allows longitudinal recordings, lets us amass a huge amount of data from a relatively small number of animals and means that statistical tests are more powerful as an important source of variability is reduced. Thus, data output will be maximised and variability will be minimized. In addition, during in vivo electrophysiology experiments multiple neurons can be recorded from a single animal, which increases the yield from each experiment significantly. Finally, potential mechanisms will be examined, where appropriate, in ex vivo preparations where multiple measurements can be obtained from a single animal, for example by taking several brain slices.

We follow best practice with respect to principles of experimental design and wherever possible use blinding, random group allocation, and objective analysis techniques to minimize bias. Specifically we will implement the Experimental Design Assistant (EDA) for visualising our experimental design for facilitating a more robust review of the experiments design which includes the power analysis used to determine animal numbers, experimental layout, data analyses and how these feed back into these and related experiments. All data will be recorded so that publication can follow ARRIVE guidelines.

Genetically-altered mice and rats will be used. Efficient colony management (which is in place at our unit) will ensure that only colonies being used will be mated to produce animals. Those no longer in use will be cryopreserved, if not already preserved elsewhere. In addition, we will use both male and female rodents and attempt to use wild- type littermate animals as controls, whenever possible, or excess wildtypes will be used in other experiments not requiring transgene expression.

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 and mice are very well-characterised species, especially in this type of study, and so there is a broad background of prior research to base techniques and hypotheses on. Work in both species is complementary as a number of techniques can only be effectively performed in one species. We will combine a literature-based with an experiential approach where for any new technique or behavioural protocol we will base our experiments on a thoughtful consideration of all the relevant literature and the objectives of our study. For techniques and behavioural paradigms we have previously implemented the methodology will form the basis of out study but will always be considered and updated in light of recent published work and the 3Rs.

Our behavioural paradigms are chosen to resemble natural food-seeking behaviour where animals learn that a series of actions result in a food reward. We are constantly refining our behavioural experiments and recently we have optimised the conditions so that the same animal can be effectively used across different behavioural paradigms. A mild food restriction during the task helps in facilitating the learning and engagement within such tasks and animals are always given their full daily food amount immediately after their daily behavioural experiments. During food restriction, animal weight and general health (activity levels, appearance, etc.) is monitored daily. The lab has a good deal of experience with intracranial surgeries and cranial implants which have been refined throughout the years. For example, we have optimised the use of skull screws and have updated the type and amount of adhesive used for stabilising the implant.

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

Rats and mice will be used as the least sentient species appropriate for this type of work, some of which involves invasive recordings/manipulations in awake, behaving animals.

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

- Food restriction will be minimised with animals encouraged to perform tasks through use of an appropriate feeding schedule (e.g. feed in afternoon, test following morning) rather than severely restricting the amount of food available.
- Our procedures for constructing head caps and implanting devices were refined over the lifetime of the previous licence in particular using a different, stronger cement. We will continue to use these refined techniques and have shared this practice with other researchers locally and further afield.
- Animals will have environmental enrichment and will be group-housed. For animals with head implants, where rigid tunnels may interfere with implants, alternative enrichment will be used such as cardboard houses.
- Animals will be handled appropriately using refined methods (e.g. not by the tail) and for mice with head implants, when tunnel handling is inappropriate, cupping will be used to remove them from the cage.
- Appropriate anaesthetic/analgesic regimens will be used to minimize pain e.g. delivery of pre- operative analgesia.
- Maximum injection/infusion guidelines will be adhered to.
- Non-Schedule 1 methods of killing will only be used under terminal anaesthesia. These methods will be used to ensure that the quality of extracted tissue is appropriate to meet the scientific aims of the study.

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

We will regularly monitor the NCRs webpages for updates in best practice in animal behaviour experiments and aseptic techniques and post-operative care. This will include following published work on welfare assessment, anaesthesia, analgesia, rodent surgery and following NC3Rs "procedures with care" resource and IMPROVE guidelines. Moreover, all experiments will be designed according to the ARRIVE guidelines 2.0 and all work will be published in line with this framework.

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

My group will regularly visit the NC3Rs webpages for updates in ways to incorporate the 3Rs in our research. These web pages also announce upcoming 3R-related events which can now be joined online. The plan is to join some of the events but also to suggest to students and postdocs in the lab to do the same. In addition, I will be in close contact with the facility management and staff but also the resident veterinarian so as to be better informed on how to implement any new 3R policies into our research.

6. RNA Therapeutics for Musculoskeletal Disease

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

Inflammation, Arthritis, Pain, Treatment, Gene editing

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 evaluate and optimise a novel form of gene therapy – gene editing – for treating patients with inflammatory and degenerative diseases affecting the musculoskeletal 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?

Musculoskeletal diseases such as osteoarthritis, rheumatoid arthritis and low back pain are important causes of morbidity, reduced mobility and lost working days in adults.

Osteoarthritis (OA) affects 7% of the world’s population - over 528M people. A diagnosis of OA is also associated with a 50% increase in the risk of heart disease. It is now the third most common cause of chronic disability, behind diabetes and dementia. The direct costs of OA include expenses related to medical treatments, surgeries, and medications, while

the indirect costs arise from lost productivity, absenteeism, and disability. The total annual cost of OA is estimated to be $136B in the United States and approximately £10.2B in the UK.

Low back pain is also a common health issue with substantial economic implications. The direct costs associated with low back pain include costs from medical treatments, diagnostic procedures, rehabilitation, and medications. Indirect costs, which are even more onerous on society, come from reduced work productivity, missed workdays, and disability. The total annual cost to the US economy of low back pain is variously estimated at between $50-100B and the equivalent cost to the UK economy is estimated at £12.3B.

Our primary research focus lies in the development of novel molecular therapies to treat or prevent pathology in the context of these (and other) common musculoskeletal diseases. Our approach involves the use of cutting-edge gene editing using the CRISPR-Cas9 system, which is an adaptation of the genome editing system found naturally in bacteria that protects bacteria from invading viruses. We are using the CRISPR-Cas9 system to selectively block the release and/or biological effects of chemical factors that have been implicated in inflammatory, degenerative and neoplastic disorders of the musculoskeletal system. Our initial focus for this license application is on inflammatory and degenerative joint diseases such as gout and OA.

The programme of work outlined in this license focuses on increasing our understanding of how RNA therapeutics can be used to treat or prevent these diseases. Results will define a pathway forward to regulatory approval and clinical delivery of this new class of therapeutic agents in human and veterinary patients.

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

The main output of this project will be new information about how gene editing can be successfully applied to animal models of musculoskeletal disease, paving the way for clinical translation into human and veterinary patients with naturally occurring disease. We anticipate several important outputs from this work, including new information about disease mechanisms and pathways, insights into the safety and efficacy of novel delivery solutions, and preclinical data that will be used to support regulatory filings with the Food and Drug Administration (FDA), the European Medicines Agency (EMA) and the UK Veterinary Medicines Directorate (VMD).

With respect to mechanistic outputs, our experimental work will characterise a range of disease models through a combination of genomics (evaluating the DNA itself), transcriptomics (evaluating gene expression) and proteomics (measuring protein production). These experiments will validate our choice of initial targets for therapy, but we also expect to identify novel targets in these experimental systems. The clinical relevance of these targets in natural disease states will then be assessed in biological samples derived from human and veterinary patients, as appropriate.
Our work on novel delivery systems for transferring RNA species into target cells will focus on the development of robust *in-vitr*o (cell culture), *ex-vivo* (explant tissue) and *in-vivo* (whole-animal) models to recapitulate the biology of musculoskeletal disease and to predict the performance of novel delivery systems, including the use of targeted delivery systems. Taken as a whole, this work will allow us to screen large numbers of candidate delivery systems and select only those with biological efficacy for future translation to the clinic.

The results from our work will be presented to learned societies, published in peer-

reviewed journals and made available to regulatory bodies such as the FDA, EMA and VMD as part of the approval process for new therapeutics. We expect that the data will be used to support the development of clinical trials in both humans and animals (including dogs with OA).

In the long-term, we would hope that our work will lead to radically improved local treatments for serious and prevalent musculoskeletal conditions in humans and animals around the world.

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

In the short-term, the main beneficiaries of our work will be researchers, both in academia and the pharmaceutical industry, and the general public through our public engagement work. Effective communication with the lay public is critical, not only to educate and inspire the next generations of clinicians and biomedical scientists, but also to demonstrate the value of the research that is supported through UKRI and UK-based charities.

In the long-term, beyond the time of this project, we anticipate that findings from our research will lead to the development of innovative therapies to prevent and / or treat inflammatory, degenerative and possibly neoplastic diseases of the musculoskeletal system.

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

We will disseminate results through standard scientific channels (conference presentations and publications, including the use of open access publications such as F1000Research). Our work on the development and refinement of screening models for predicting delivery will be especially relevant to the field of RNA therapeutics. The work outlined under this license will also support the training of a PhD student, whose work will be presented at meetings and published in peer-reviewed journals.

Species and numbers of animals expected to be used

- Mice: 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.

We are using skeletally mature mice (12 weeks of age or older) for this project. Skeletally mature animals are used because the diseases that are being studied are much more prevalent in adults compared to children or adolescents. Mice are preferred over larger animals because they are inbred, allowing for more precise control over patient variables that could otherwise complicate the interpretation of study results. The use of mice is also preferred because of the existence of several validated models of joint disease in mice, including models that involve injection of chemicals into the knee and surgical models that cut one or more of the ligaments that stabilise the knee (either the medial meniscal ligament or the anterior cruciate ligament). Given the utility of the mouse as a preclinical model for OA, and the fact that we would evaluate candidate therapies in this model, it is logical that screening studies needed to optimise mRNA delivery to the joint would also be

performed in mice.

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

The animals used for safety/efficacy testing of novel delivery systems are not expected to experience pain or distress greater than that associated with an injection. Joint injections would be performed under general anaesthesia to eliminate any pain/distress associated with the intra-articular injection. Post-operatively, mice will be imaged to track the delivery of the mRNA and then they will be killed humanely.

For the inflammatory joint disease model, mice will be pre-treated with a therapeutic (CRISPR construct or a clinical treatment, such as steroid or hyaluronic acid, that is already approved for use in humans and/or animals) and then challenged with monosodium urate crystals (the crystals that cause joint inflammation in patients with gout). We will assess the local tissue response by measuring joint width, pain and limb use. In some cases, mice may be challenged with MSU up to two more times to see how long the protective effects of the treatment last. At the end of the study, mice will be killed humanely.

For the surgical models of OA - mice undergoing destabilisation of the medial meniscus (DMM) or ACL transection (ACLT) - will undergo an single surgical procedure in which one of the small ligaments in the knee (stifle) joint is cut. After an initial period of lameness associated with joint inflammation (synovitis), the mouse will develop progressive changes in the soft tissues (joint capsule), cartilage and bone that mimic those seen in patients with OA.

Surgery will be performed with the animal under general anaesthesia to ensure that pain and/or distress are mitigated. Post-operatively, animals will be treated with clinically approved drugs (e.g. analgesics, antibiotics, fluid support, etc.) and recovered in pre-warmed recovery cabinets. The mice will be observed for periods of up to 16 weeks, at which time they will be killed humanely.

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

Mice injected with novel candidate lipid delivery vehicles are not expected to experience pain or distress beyond those associated with an injection. We would not give analgesics to these mice as a matter of routine but will do so if the mice develop a non-weight-bearing lameness. Should animals experience pain that is not responsive to analgesics, they will be killed humanely.

For the MSU injection model, where we want to assess the effects of the new therapeutic on acute joint inflammation (synovitis) and pain, we would not use analgesics as they would mask the effect of any medication, making it impossible to determine whether the new treatments are beneficial. The effects of MSU injection are transient and self-limiting (12-24 hours) without treatment; any animal that demonstrates a non-weight bearing lameness will be treated with analgesics. Should animals experience pain that is not responsive to analgesics, they will be killed humanely.

Human and veterinary patients with inflammatory or degenerative joint disease experience a variable degree of pain and functional impairment. The same is true for rodents. For the DMM and ACLT models, we anticipate that most of the inflammation and pain will be seen in the first 3-5 days after surgery. Since the goal of these experiments will be to look at the

effects of the new treatment on OA pain, which does not develop for at least 2-3 weeks after surgery, we would administer analgesic drugs for the first 3 days after surgery, then reassess animals. Anti-inflammatory drugs such as steroids or non-steroidal anti-inflammatory drugs (such as aspirin, meloxicam or carprofen) will not be used as they will interfere with the inflammatory pathways that we are studying and targeting with our therapeutics. Should animals experience pain that is not responsive to analgesics, they will be killed 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)?

There are no severe protocols associated with this project.

Animals receiving intra-articular injections of the therapeutics, mRNA beacons or vehicle controls (**Protocol 1**) are not expected to develop any significant tissue response. Intra-articular injections are performed routinely in human and veterinary patients, typically without sedation or general anaesthesia. Under this license, we will perform all of these procedures under general anaesthesia so that the animal is immobilised (to reduce the risk of inadvertent injection outside the joint if the animal moves unexpectedly) and to ensure that there is no pain or distress associated with the injection itself. We anticipate that >80% of these animals will experience mild severity, no greater than the pain or distress of an injection; animals that develop mild or moderate joint swelling or lameness would be treated with clinically approved analgesics, as outlined in the later sections. Should animals experience pain that is not responsive to analgesics, they will be killed humanely.

For the mice being challenged with MSU and/or LPS (Protocol 2), we anticipate a mild to moderate tissue response and associated joint swelling in ~100% of animals. These studies would be classified as moderate severity for this reason. Animals injected with the saline vehicle would not be expected to reach moderate severity. Overall, approximately 80% of mice from **Protocol 2** would likely meet the moderate severity

For animals on the two surgical protocols (**Protocols 3 and 4**), mild to moderate lameness and discomfort is anticipated within the first 3-5 days due to early inflammation from surgical trauma, followed by slow but progressive damage to joint tissues. Over the course of the full experimental period, 75% of mice that have undergone DMM or ACLT would be expected to reach moderate severity. Regular pain scoring will be undertaken to identify and limit the pain experienced by an individual animal, and objective humane endpoints have been defined for each animal model.

Table 1. Anticipated severities for mice under this project licence application.

Protocol	Mild	Moderate	Severe
1	80%	20%	0%
2	20%	80%	0%
3	25%	75%	0%

4	25%	75%	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?

This research will investigate complex, whole-organism disease processes, such as inflammation and joint destruction, and the effects of molecular therapies given by local injection into the joint. To study these processes, it is necessary to study whole, intact organisms because of the interactions that take place between different body systems to drive disease development e.g. the resident cells of the synovium (the inner lining of the joint space), the chondrocytes (cartilage forming cells) in the articular cartilage, and the inflammatory-immune cells that are delivered into the joint from the bloodstream.

Moreover, to correlate our work with humans experiencing similar conditions and to thus aid clinical translation, it is necessary for us to study how an underlying pathology affects an animal's behaviour, which can only be done if whole animals are used. For example, humans with joint pain are less active due to activity-induced pain and we use similar assays to measure how arthritis and other conditions in mice affect their natural behaviours and their use of the affected limb(s).

Studying less complex organisms would be inappropriate because they do not have the necessary physiology that would enable us to simulate human pathologies. For example, arthritis is a disease of the joints and requires animals with synovial joints. For our work, rodents reflect the lowest order animals that are appropriate for the questions being addressed.

As described below, we are using a suite of computational tools and laboratory (*in-vitro)* screens to identify potential candidate therapies, without the need for initial animal testing.

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

Although we are now seeking licence authority to undertake studies in live animals, our therapeutics are developed rationally through a combination of computational (*in-silico*) and cell culture (*in-vitro*) tests to ensure that only viable drug candidates make it to animal testing. By referring to published data on the mouse and human genome, we have been able to make use of robust computational tools to predict the performance of the gene editing therapy. It is not uncommon to screen over 1000 candidates *in silico* and to select only the top 10-20 for ongoing analysis. This more limited list of candidates is then screened *in vitro* to determine biological efficacy, and the best 2-4 therapeutics per gene target are then moved forward to animal testing.

Why were they not suitable?

Computational approaches are ideal for screening through 1000s of compounds to identify

top "hits" that might interact with a target of interest. The *in-silico* models are excellent at predicting interactions between CRISPR machinery and the mouse genome, but they cannot account for variations in CRISPR delivery, the effects of the immune system, or the pharmacodynamics of the CRISPR molecules in joint tissues.

The *in-vitro* systems that we use are similarly excellent for confirming the biological effects of the mRNA on cells, but they do not account for variations in delivery efficiency that are known to exist *in vivo*. For example, it has been shown that delivery into soft tissues such as synovium is more straightforward than delivery into dense matrix tissues such as cartilage or meniscus. In a similar manner, cell culture models with isolated cells cannot recapitulate the complex cell-cell networks present in composite tissues such as the synovium, where multiple cell types are known to exist. Some of these limitations may be addressable with mixed cell culture models or cultured organoids, but these are relatively nascent fields in musculoskeletal biology. Finally, nothing short of the whole animal can currently define the pharmacodynamics of a material injected into the knee joint. This is critical to the success of candidate intra-articular therapies.

We are actively working to develop improved predictive models for assessing the tissue-level effects of CRISPR therapeutics through the use of explant culture models – when fully deployed, these methods will be used as a second-level screen for candidate therapies, but they will not fully replace the need for confirmatory live-animal studies that define the timing, tissue distribution and biological consequences of a CRISPR-based edit.

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 analyses will be employed ahead of starting experiments to ensure that the minimum number of animals will be used, as is necessary to reach experimental objectives. Such analyses will be driven by our own prior experience (e.g. with joint injection models), advice from colleagues familiar with the models, and data from the wider scientific literature.

In addition, where appropriate, preliminary (pilot) experiments will be carried out in a small number of animals to assist with experimental design and refine group size estimates. For example, to determine the efficiency with which a new delivery system supports RNA transfection, small numbers of animals will be used for a dose escalation study, and the optimal dose would then be the only one used in subsequent (larger) studies.

Group sizes for our experiments are typically in the range 8-12 animals per condition and time point, depending on the model being used. The single greatest driver of total animal use will be the number of therapeutics evaluated under this license - over the next 5 years we expect to develop a minimum of 8 new therapeutics for evaluation in mouse models.

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

We keep up with the latest literature to make sure that we are only conducting experiments that are scientifically appropriate and to prevent the meaningless repetition of studies conducted by others elsewhere.

Where possible, we conduct computational and cell line work before proceeding to using animals. For example, we use computational modelling to predict the best candidates for the guide RNA (gRNA) that serves to target the CRISPR therapeutic to the genome. We then validate the most promising of these guides in multiple cell culture models to confirm the biological relevance of the resulting edit to the genome. We only use live animals when we have compelling data to demonstrate a biological effect. We make use of the NC3R's Experimental Design Assistant to confirm the validity of our experimental design, and will consult with a biostatistician in the early stage of each study in order to ensure optimal experimental design and rational use of animals.

For studies involving joint surgery we will make use of "Sham controls" (mice that undergo a surgical incision to expose the joint but that do not then have the ligaments cut). Wherever possible, we will use the same Sham controls across multiple experiments, avoiding the need to do individual Sham groups for both procedures.

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 used are inbred to produce more reliable results (inbreeding reduces the genetic variation and thus limits one possible source of variation in experimental measurement) resulting in a lower overall number being used.

We regularly design experiments to allow for a combination of in-life and post-life outcome measures from the same animal. For example, mice receiving intra-articular injections of candidate therapies will be followed in-life with bioluminescent imaging (to define the temporal and spatial pattern of mRNA delivery and expression) and then we will use techniques such as single-cell RNA sequencing and spatial transcriptomics to correlate tissue distribution with the presence of an CRISPR-based edit.

Members of the research team subscribe to an internal tissue sharing scheme, which supports sharing of animals, techniques and resources locally.

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 mice is preferred because of the existence of several validated models of OA in mice, including direct injection of chemical irritants and surgical models that involve cutting of one of the ligaments inside the knee joint - either the medial meniscal ligament (leading

to destabilisation of the medial meniscus, also known as the DMM model) or the anterior cruciate ligament (knowns as the anterior cruciate ligament transection, or ACLT, model). Given the utility of the mouse as a preclinical model for OA, and the fact that we would evaluate candidate therapies in this model, it is logical that screening studies needed to optimise mRNA delivery to the joint would also be performed in mice.

Many of the models we use simulate very closely the disease in patients. For example, the ACLT model produces a disease profile that is very similar to that seen in human and canine patients that experience an ACL rupture – immediately after the injury there is an acute inflammatory response, with joint swelling and lameness; this subsides and then a slower, progressive process of chronic soft tissue inflammation and cartilage damage that ultimately leads to the demise of the joint. In humans, the result is an arthritic joint that will require medical or surgical management. In the mouse model, our study is designed so that animals come off study before they experience significant pain or distress from the arthritic joint.

The primary goals of our intra-articular therapies are to limit inflammation and cartilage damage. For this reason, we cannot use drugs that are known to modulate these processes *in vivo*. This means that the systemic use of anti-inflammatory drugs such as meloxicam, aspirin and corticosteroids is contraindicated in our studies. We will however make use of alternative approved analgesics to limit post-operative pain in the surgical models, and the end points of the study are designed so that they allow for the development of detectable radiographic and microscopic evidence of joint pathology with minimal clinical symptoms.

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

In this project we will study how mRNA therapeutics modulate inflammation and joint damage. This work involves the use of an organism with a synovial joint, innate and adaptive immune response, and a nervous system that allows us to study the behavioural effects of joint disease and its treatment.

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

In general terms, the welfare impact on the animals will be minimized through use of:

1) Clinically approved anaesthetics, analgesics and, when clinically indicated, antibiotics.
2) Consultation with the Named Veterinary Surgeon.
3) Aseptic surgical technique.
4) Careful monitoring in the immediate perioperative period, provision of supplemental heat and nutrition as needed.
5) Routine use of group housing and environmental enrichment.
6) For animals experiencing conditions lasting several days or weeks, they are regularly monitored (e.g. assessing limb use and measuring joint width for animals experiencing experimentally induced inflammation) and weighed. We also plan to evaluate new refinement methods, such as monitoring cages, to further refine our methods for assessing recovery after surgery.
7) We will make use of palatable substances such as pastes or jelly to facilitate the administration of oral substances such as analgesic drugs.
8) In addition to pain management, we will take steps to enrich the animals’ environment as appropriate, e.g. providing food in the form of mash (available on the cage floor to prevent the need for animals to rear up to obtain food), and providing supplemental

heat through the use of cage warmers in the early post-operative period.
Lastly, we will use non-aversive handling techniques where possible, and always allow acclimatisation of the animals when brought into the facility before starting a study.

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

We will conduct our research according to the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines and make sure that we report our findings in line with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

We will also use online resources, such as the humane endpoints webinar (www.humane-endpoints.info/en), NC3R's resources for breeding and colony management in genetically altered mouse colonies (www.nc3rs.org.uk/3rs-resources/breeding-and-colony-management), and NC3R's experimental design assistant (www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda).

Lastly, we will keep up with the latest scientific literature to monitor advancements in the field about how to look after our animals and conduct experiments in the most refined way.

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

We will take the following steps:

1) Routinely check the NC3R's resource library and keep abreast with, and implement where appropriate, any new advances in refinement, reduction and replacement that are relevant to our project.
2) Consult guidance from the Laboratory Animal Science Association (LASA), Institute of Animal Technology (IAT), and the Royal Society for the Prevention of Cruelty to Animals (RSPCA) that provide additional sources of new recommendations and advances in animal techniques and clinically applicable models.
3) Have regular discussions with the Named Persons and animal technicians within the facility to review current approaches and whether there are any new 3Rs opportunities.
4) Participate in an internal tissue sharing scheme, which also provides an opportunity to keep up to date with 3Rs news and events, as well as take advantage of opportunities to share tissues and knowledge.
5) Attend NC3R's workshops where appropriate and monitor publications in journals, such as Laboratory Animals and Lab Animal to stay abreast of the latest developments in all aspects of the care and use of animals in biomedical research.

7. The phosphoinositide network in health and disease

Project duration

3 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

Phosphoinositides, Cancer, Metabolism, 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 aim to better understand how a wide-spread cellular signalling network that works inside cells, called the phosphoinositide network, functions and controls diverse and essential processes like metabolism, the rate of ageing and tumour progression.

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 impacts of ageing are both a direct loss of overall robustness with age and also a big increase in susceptibility to a variety of diseases that are known to be more frequent in the elderly, such as cancer, metabolic disease, arthritis and dementia. The increasing proportion of global and UK populations that are elderly is a major social challenge. Our work aims to find some of the molecular reasons why and how there is loss of function and

increased susceptibility to disease in the elderly and if there might be connected explanations for the emergence of cancer and metabolic disease in some people. This body of work has the potential to define precise targets for new medicines to slow or reverse these processes.

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

Publications, patents, new knowledge and the early stages of developing new clinical approaches to slow or reverse metabolic disease, tumour progression and ageing.

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

The UK will benefit from the work and the potential biomedical investment flowing from our outputs. The scientific community and commercial sector will benefit from increased knowledge and understanding in the short to medium term. In the longer-term patients will see improved health outcomes resulting from better strategies to treat age-related metabolic diseases such as diabetes and also more selective drugs, with fewer side-effects, to treat cancer generally and prostate tumours particularly.

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

We collaborate with, and disseminate knowledge to, academic and commercial colleagues. We publish our findings when they are both positive and negative. We engage with the public through a variety of means including presentations or installations at Science Festivals. We host local and international students and schools' projects to explain our work.

Species and numbers of animals expected to be used

- Mice: 11,980

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 exclusively use mice. They are a very well validated model for many mammalian processes that are important in humans including ageing, metabolism and cancer progression. Our planned use of mice will enable us to use directed specific genetic modifications and potential medicines to determine how the phosphoinositide network operates and controls ageing, cancer and metabolism and whether we can modulate the network to improve health. These things cannot be done with humans or human cell lines because of ethical considerations or because of the fact cell lines cannot recapitulate many aspects of cancer progression, metabolic disease and ageing that are seen in whole mammals. Other than creating and maintaining genetically modified mice our work will only use adult and elderly mice.

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

The large majority of animals used are used for breeding and maintenance of genetically

modified strains that have no or very mild phenotypes when they are looked after inside a carefully run specialised animal-facility.

- A number of mice will require ear biopsies for genotyping.
- A very small number of mice will require recovery surgery.
- A small number of mice will be aged, up to a maximum of 24 months, and will be watched especially carefully to ensure that no unexpected health problems emerge and will be humanely culled.
- A small number of aged or young mice will be fed modified diets, for up to a maximum of 20 weeks, that will lead the mice to become over-weight and experience metabolic disease a bit like diabetes, they will be closely observed so that no unexpected or significant health problems emerge and will be humanely culled.
- A small number of young and aged mice will receive a small dose of sugar by injection into the peritoneum (belly) to test their metabolic health.
- A small number of aged and young mice will receive an injection (either under the skin on the flank or into the peritoneum) of insulin to test their metabolic responsiveness
- A small number of mice will be bred to have a combination of genes that will lead to the slow development of prostate cancer and will be observed very carefully to ensure that the mouse does not experience significant symptoms, will not be allowed to progress beyond 16 months, and will be humanely culled.
- A small number of mice will be injected with tumour cells under the skin of the flank. The mice develop small tumours relatively rapidly but will be culled as soon as a reliable measurement of the rate of tumour progression can be made, much previous work shows this varies between 2 and 3 weeks depending on the tumour cell line. The well-being of the large majority of these mice is not impacted by the tumour.
- Potential medicines we use to treat a small number of mice, or further genetic modifications we introduce into mice, we expect to reduce or stop the ageing process, the emergence of metabolic disease or prostate cancer development.
- No mice will be reused in experiments.

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

For most of the mice, including immunodeficient strains (that is mice with a specifically weakened immune system), we do not expect any impacts or adverse effects in our high-quality specific pathogen-free (SPF, that is environment without any disease-causing microbes) animal care facility. NOD SCID Gamma (NSG) immunodeficient 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.

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 diarrhoea, eye abnormalities, abdominal swelling 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.

Tumour progression following sub-cutaneous (under the skin) inoculation with tumour cell

lines will lead to emergence of a tumour with a humane end point being reached within a maximum of 3 weeks. For some cell lines it will be quicker. Progression is consistent (does not vary significantly between mice and normally has no impact on the well-being of the animal.

Tumour progression in models of endogenous mouse prostate tumour progression following conditional loss of PTEN (a tumour suppressor gene, the loss of which causes helps cancers grow) is very slow and mice do not experience any impact on their wellbeing as a result of the genetic alteration for the first 6 months. The majority of our mice will be culled before they reach 6 months. A small proportion will be allowed to progress to a maximum of 14 months. In this model there is no evidence that the tumour spreads to other tissues and the large majority of animals will reach 14 months without exceeding mild signs. About 2% of mice may display moderate 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)?

Subthreshold 75.1% (9000). Mild 23.1% (2764). Moderate 1.8% (216).

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?

Studying how the phosphoinositide network (a ubiquitous intracellular signalling system) regulates the rate of ageing, metabolism and tumour progression, with the aim of improving patient outcomes in these areas, must entail use of animal models of these processes as they are all based on the organismal level cooperation between cells and tissues in a way that cannot be replicated in vitro or in silico. Where possible we have and will use cell culture and organoid systems to substitute for experiments with animals. Systems biology approaches based on computer modelling of signalling, including the phosphoinositide network, are now being applied and our lab has used these techniques to make important advances, however, none of these models is capable of dealing with the many interactions between tissues and different cell types that occur during ageing and tumour progression for example.

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

We are using mouse-derived prostate organoids, human prostate-derived cancer cell lines and many other cultured cell lines as well as computational approaches to better understand how the phosphoinositide network operates at a cellular level. The results from these past and on-going experiments contribute to the evolution of our hypotheses and

models that aim to understand how the phosphoinositide network functions in vivo. However, our results show that the phosphoinositide network does not function the same in mouse prostate organoids as it does in vivo and further that it does not function the same in cultured cells as it does in organoids.

Why were they not suitable?

Our published results show that the phosphoinositide network does not function the same in mouse prostate organoids as it does in vivo and further that it does not function the same in cultured cells as it does in organoids.

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 mice required for the generation of modified mouse strains are based on the standard operating procedures extensive experience and literature review. The numbers of mice required for the breeding and maintenance protocols is based on estimations of mouse strain numbers, experience at sustainable colony management practice and the frequency of required genotype combinations. The numbers of mice required for individual experiments are based on power calculations and statistical modelling. We input the known statistical properties (phenotype average and variation), decide upon the minimal effect size acceptable from the experiment from a biological perspective and hence calculate the appropriate group size. The number of experiments required within each protocol is based on the assumption of all go-no go decisions being positive and successful grant funding achieved.

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

We use the NC3Rs Experimental Design Assistant where possible and take advice from the our organisation's statistician about the most effective ways to achieve statistical power without increasing the number of animals used. We work to minimise variation where and when possible. This includes ensuring related experiments are conducted at the same time in the day every time, so that we ensure mice are at the same point in any night/day cycles in their behaviour and metabolism.

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 efficient breeding programmes that are fitted to the numbers and frequency with which mice are needed for experiments. We take multiple samples from mice to ensure we can repeat assays if needed, share their tissues and be used for other assays to be performed. We use pilot studies to ensure that our analyses sample biological effects in the optimal context (e.g., dose or time of exposure).

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 are advantageous for biological discovery. They are small and easy to breed, reaching sexual maturity within two months from birth, and have the capacity to produce large numbers of embryos. Furthermore, mice are the best animals for our research because they can provide all the genetic modifications we require. Our gene alterations aim to change the way the phosphoinositide network operates to allow us to understand its role in ageing, metabolism and tumour progression and we do not anticipate they will produce any unexpected deleterious outcomes for the mice.

Most animals on this project are not expected to be subject to any pain, suffering, distress or lasting harm.

Some of the animals (<5 %) in the project will be maintained for a long period of time to investigate ageing. There are no specific impacts or adverse effects expected on mice ageing healthily during this project, and regular monitoring of aged animals will prevent for any unnecessary animal distress.

To study metabolism we will use extremely well validated and understood methods.

The majority of our studies of tumour progression will end before the mice experience any observable signs of disease. The small proportion that allow further tumour progression will be closely monitored and experiments will end before disease progression reaches levels set by international guidelines for studying cancer in mice.

Animals will be habituated to being handled and weighed regularly during protocols to study acute metabolic challenge (6) and tumour progression (7 and 8).

For novel substances and/or routes, a pilot study of a small number of animals would be performed with input from the NACWO (Named Animal Care and Welfare Officer) and NVS (Named Veterinary Surgeon).

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

The choice of species is limited by the fact that invertebrate species do not have a phosphoinositide network that is comparable to that in humans nor do they age, regulate their metabolism in the same way and experience related tumour progression processes.

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

We will follow the latest advice for specific procedures, and we will bring in external

experts when needed to refine our methods. We will listen to feedback from experienced PIL (Personal Licence) holders running the experiments, particularly with regard to monitoring and welfare.

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 (e.g., the ARRIVE guidelines and aim to work to the PREPARE guidelines (norecopa), the LASA aseptic guidelines, LASA Diehl guidelines on volumes and frequency limits (Diehl et al. A good practice guide to the administration of substances and removal of blood, including routes and volumes. 2001 J. Appl. Toxicol. 21, 15-23) and the most up- to-date veterinary knowledge. We work to the HO guidelines for efficient breeding. In our work with mouse models of cancer we will follow guidance on end points as described in 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.

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

We will actively stay updated with our field of research through collaboration, conference attendance and reading the literature. We will take particular note of any technical advances that enable reduction, refinement or replacement in our experimental design. The local mouse facility is also a key source of knowledge, transmitting the latest information on the 3Rs to researchers. Internal protocols are shared across the organisation, enabling rapid uptake of any improvements to the method across groups.

Our NIO (Named Information Officer) is pro-active in sharing 3Rs updates on a monthly basis through a newsletter.

8. Assessing the duration of antibodies to Schmallenberg virus in naturally infected sheep

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

Schmallenberg virus, Epidemiology, Immunity, Congenital abnormality, Insect vector

Animal types	Life stages
Sheep	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?

Schallenberg Virus (SBV) is a recently emerged virus which causes outbreaks of foetal deformities in cattle and sheep in the UK and across Europe. Outbreaks of the disease occur every 4 to 7 years and are usually unexpected and can be severe, causing large outbreaks of deformed lambs and calves and very difficult births for the ewes and cows. The unpredictable nature of the outbreaks make planning for prevention very difficult for vets and farmers. Outbreaks are hypothesised to be caused by the presence of virus circulating in the midge population when animals are pregnant and, either their immunity from natural exposure has declined to SBV, or, they are immunologically naïve to SBV. However, this is not fully understood.

The aim of this project is to measure the presence and duration of protective immunity to SBV, in a five year, longitudinal, sero-prevalence study of a naturally SBV infected, commercial sheep flock; whilst concurrently measuring SBV infection in the farm’s midge population.

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 outbreak years SBV can cause major impacts on animal welfare. This is primarily because of difficult births for the mothers and injury and death to the offspring; with early season lambing flocks being particularly, badly, affected. Some flocks report up to half their lambs have been lost in 2024 due to SBV. Economic analysis demonstrated a loss of between £6 and £20 per ewe in a flock in an outbreak year and, understandably, SBV has a substantial impact on farmer's well-being. If we can understand why the outbreaks of disease occur, farmers and vets can plan disease prevention through use of vaccines and changes in their management.

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

The output will be a disease outbreak prediction model. This new information on the epidemiology of Schallenberg virus which will be used to help inform disease prevention strategies such as timing and groups of animals for vaccination. These will be published in scientific publications and shared with farmers and veterinary surgeons through farmer meetings, webinars and publications.

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

The work will impact on farmers, veterinary surgeons, pharmaceutical indutry within 12 months of completion of the project.

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, webinars and press articles.

3) We will collaborate with farming industry bodies, such as Agriculture Horticulture Development Board, Sheep Veterinary Society, Ruminant Health and Welfare Group to disseminate the findings.

Species and numbers of animals expected to be used

- Sheep: 1500 sheep

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 be undertaken on a sheep flock which has experienced a naturally occurring outbreak of SBV this year (2023-2024). We will collect two blood samples each year from each animal in the flock to measure their immunity to SBV virus, before and after the midge season. We will monitor flock level and individual animal immunity with laboratory tests to measure their immunity changes over the 5 year period. At the same

time, we will measure the presence of SBV in the midge population on the farm. All ages of sheep in the flock will be sampled.

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

Each animal in the flock will be blood sampled twice per year. The sheep will remain in their flock for the whole period.

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

The sheep 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 the procedure 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 were naturally infected with SBV in 2023-2024. We need to understand the change in their immunity to SBV over time to help us understand and predict future outbreaks of this disease.

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?

The estimate is based on the current flock size of the naturally infected flock (approximately 500 ewes) being sampled for 5 years and 20-25% of the flock being replaced each year with new breeding females.

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

We will be sampling the all the sheep present in the flock so that we can measure what happens to flock level and individual animal level immunity.

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 electronic data identification of the sheep in the flock to allow us to monitor individuals over time and maximise the data collected 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.

Sheep, naturally infected with SBV, in 2023-2024 (prior to the start of the study) will be used. The flock will have had laboratory confirmed SBV infection; for congenitally affected lambs born in 2024, the time of infection of the mothers' is considered to be typically 1-2 months of gestation. The blood sampling procedure is brief and mild and should only cause transient discomfort to the animals.

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

Less sentient animals are not affected by SBV.

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

All animals will be monitored for at least 1-hour post sampling for any adverse effects, should these occur veterinary attention will be sought.

All procedures will be carried by a Home Office Personal Licence holder 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.

9. Investigating Hematopoietic and Immunological Approaches to Cancer Cure

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cancer, Stem cells, Immune therapy, Blood diseases, Aging

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 seeks to investigate the potential of haematopoietic (blood cell formation) and anti-tumour pathways in developing a cure for cancer. We will utilise mouse models to test novel strategies aimed at:

i) Enhancing the body's natural ability to produce healthy blood cells and prevent blood cancer

ii) Strengthening the body`s immune system's capacity to recognize and eliminate 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?

By studying the function of the cells of the body that develop into blood cells and the body`s immune responses we will gain a deeper understanding of the mechanism that cause cancer and uncover strategies to enhance treatment of cancer.

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

This research aims to understand how blood stem cells with pre-cancerous changes grow out of control in older adults. We will see how things like surroundings, medications, and inflammation affect this process. Additionally, we will develop a method to improve the immune system's T cells and test its use in battling solid tumours (cancers that form lumps). Overall, this project will result in scientific articles and a potential patent for a technology that boosts T cell-based cancer treatments.

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

All areas of life sciences research, including aging research, developmental biology, cancer biology and blood biology are likely to benefit from our research. The understanding of how the cells of the bone marrow develop into blood cells (stem cells) during aging can offer insight into changing lifestyle and nutrition in the general population to counteract the switch from a normal cell into a cancer causing cell. The potential benefits of cancer cell specific therapy can be translated into the clinic via collaboration with industry partners and come to the benefit of many cancer patients suffering from solid and liquid tumours. The impact on life science research, commercial partners, and patient groups may occur in the short-term as well as many years from now.

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

I will participate and encourage lab members to engage in scientific discussion at several different levels; i) I will work to publish our results in well-known scientific journals with the highest possible impact. This will ensure maximum visibility for researchers, promote collaboration, and aid the lab in securing additional funding for continuing the research. ii) Myself or a lab member will regularlyparticipate in national and international scientific meetings to discuss our ongoing research and present results. I believe the exchange of ideas at these events is mutually beneficial and that it will have a positive effect on our research. iii) We will participate actively in the local scientific community. This includes presenting and giving feedback in institutional seminars as well as daily exchange of ideas and technical advice with colleagues at all levels. I also recognise the importance of promoting awareness of research through public outreach and providing easily accessible information to the public, and I will actively pursue public engagement in collaboration with the public engagement officer at the establishment.

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.

We will be using adult animals from in-bred transgenic mouse strains. Mice are a well-established model for studying haematopoiesis (blood cell biology) and immune function. We will utilise non-animal in vitro models where possible to understand the basic science before an animal is used. However, these models are not sufficiently complex to replicate these processes in a whole organism. Finally, the use of adult mice minimises developmental confounds and ensures a robust immune system for study.

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

The procedures in this project can be divided into the following sections:

Breeding and Maintenance of Genetically Altered Mice

1. Transgenic mouse strains with specific genotypes will be bred and maintained.

2. One strain exhibits a developmental phenotype whereby the facial features do not form normally in less than 10% of animals and will be closely monitored for adverse effects. Humane endpoints have been determined.

Adoptive Transfer to transfer bone marrow stem cells from one animal to another whose body has been depleted of bone marrow cells to study the functional role of those cells in the development of blood cells.

1. Mice will be exposed to a dose of radiation and a preventive antibiotics treatment, similar to the effects of cancer treatment in humans to enable engraftment of donor blood cells.

2. Cells isolated from the bone marrow from donors (humanely culled genetically altered mice) will be isolated and transferred into recipient mice via injection into the tail vein. These cells will then develop and grow in the mouse and the specific effects of a genetic alteration in the cells can be studied.

3. Mice with successful engraftment (the transferred bone marrow cells develop in the recipient animal) will be monitored for up to 6 months to study the function and development of different types of blood cells from each genetically altered mouse.

Anti-tumour Immunity to study the functional role of engineered white blood cells, T cells, to kill tumour cells for the treatment of blood cancer.

1. Wildtype mice will receive a skin injection of cells from a well-characterised tumour model.

2. Following tumour establishment, mice will be exposed to a low dose of radiation to deplete some cells that would target the tumour under normal circumstances as well as making it easier for the mouse to take up engineered white blood cells.

3. Engineered T cells designed for enhanced anti-tumour response will be injected via the tail vein to develop within the body.

4. Alternatively, transgenic mice, or mice with successful engraftment of mutant bone marrow (described above), will receive a skin injection of cells from a well-characterised tumour model as above but will not be manipulated further as they already contain the T cells to be tested.

5. Mice will be monitored for up to 60 days to assess tumour rejection and anti-tumour immune responses.

T cell Memory Response to stimulate the immune system to assess the overall immune function and potential anti-tumour activity of the adoptively transferred T cells.

1. Mice will receive two injections of a well-studied, attenuated Listeria bacteria strain, spaced approximately 21 days apart.

2. Mice are expected to efficiently clear the bacteria while developing a T cell memory response, that is the first dose will generate an immune response and upon administration of the second dose, the mouse will have generated the cellular response to respond to the bacteria faster.

3. The experiment will conclude 7 days after the second injection, allowing detailed analysis of the memory response.

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

A small proportion of animals with a developmental phenotype might experience health issues or discomfort such as problems with eating and drinking. Careful monitoring and adherence to humane endpoints are crucial for minimising suffering.

Adoptive Transfer

Radiation can cause side effects like nausea, fatigue, and bone marrow suppression. In most cases, these effects are temporary and will resolve within 10-12 days. Supportive measures such as soft food and warmth are provided to the affected animals.

Anti-tumour Immunity

The injection of tumour cells under the skin may cause short-term pain and discomfort.

Tumour size and animal well-being will be closely monitored, and humane endpoints will be used to determine when to stop the experiment.

T cell Memory Response

Although irreversibly attenuated, the Listeria bacteria injection might cause mild flu-like symptoms that resolve within 48 hours. Supportive measure such as soft food and warmth are provided to the affected 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)?

Breeding: Sub-threshold - 80%, Mild- 10%, Moderate - 10%

Adoptive Transfer: Moderate - 100%

Anti-tumour Immunity: Mild - 25%, Moderate - 75%

T cell Memory Response: Mild - 90%, Moderate - 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?

Mice share a high degree of genetic homology with humans, particularly in the immune system. The vast majority of genes involved in human immune response have a recognisable counterpart in mice. This allows us to study similar mechanisms and processes relevant to human health. Mice are furthermore well-established model organisms in biomedical research. Their relatively short lifespan, rapid breeding cycles, and the vast array of genetically modified strains available make them suitable for studying complex biological processes like immune function and blood stem cell development.

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

Like many scientists, we have used and continue to study human cancer cell lines to understand blood cancers. In the course of our work we will also take advantage of state-of-the-art in vitro model systems of T cells starting from human blood samples from healthy individuals.

Why were they not suitable?

Cancer cell lines are like snapshots of cancer at a later stage. While they have been helpful in studying the disease, they do not fully capture the early stages where precancerous blood stem cells become aggressive. Additionally, these cell lines lack the immune system's T cells, which are crucial for fighting cancer. This means the existing models may not fully represent how blood cancers develop and interact with the immune system. We need more accurate models to develop better treatments.

While in vitro T cell cultures starting from human samples offer valuable tools, they cannot fully replicate the intricate interplay between various organs and cell types within a living organism. Studies in a whole organism like a mouse allows us to observe these interactions in a more comprehensive manner. As such, mice remain invaluable tools for understanding complex biological processes related to the immune system and hematopoietic stem 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 estimated number of mice required for this project has been compiled based on our experience in conducting similar studies in the past, as well as review of recent publications in the field of haematopoiesis and anti-tumour immunity. Depending on the specific experimental requirements, we will use the appropriate number of breeding cages as well as experimental sample sizes that will maximize the likelihood of reaching the scientific endpoint, but at the same time reduce the 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?

We have carefully considered experimental techniques to minimise the number of animals needed per group to achieve statistical significant results. This includes: i) using existing breeding lines with desired characteristics (e.g. Ly5.1 surface receptors). ii) exploring techniques using the same animal as its own control whenever possible (e.g., having control and experimental cells within the same mouse in adoptive transfer experiments), iii) performed literature review to look for alternative endpoints. By using more sensitive or informative endpoints, this could mean that fewer animals were required to detect a significant effect, and iv) whenever data from pilot studies were available, we have used power analysis to ensure that we have enough animals to get reliable results without using unnecessary 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 explore collaborations with other researchers utilizing the same animal model to share control animals and tissue samples whenever possible. Sharing breeding stock can also significantly reduce the overall number of animals 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.

This project will utilise inbred mice. Inbred mice have a uniform genetic background, which helps to control for genetic variation that could affect the results. This is particularly important for studies involving immune responses. We will use the following models/methods:

Adoptive transfer of hematopoietic stem cells (HSCs): Isolate HSCs from donor mice and transfer them to recipient mice. This allows you to study how the engrafted HSCs contribute to blood cell formation and potentially anti-tumour effects.

Adoptive transfer of T cells: Isolate and expand T cells, potentially engineering them in the laboratory for enhanced anti-tumour activity, and transfer them to recipient mice. This allows you to investigate the role of T cells in recognising and eliminating cancer cells.

Subcutaneous tumour model: Implant cancer cells subcutaneously (under the skin) into recipient mice. This allows for the growth of a localized tumour for studying new treatments.

Listeria Monocytogenes Bacterial Infection: Use a well-characterised, attenuated (weakened) strain of Listeria Monocytogenes bacteria to stimulate the immune system in recipient mice. This can be used to assess the overall immune function and potential anti-tumour activity of the adoptively transferred T cells.

These models/methods have been selected to cause the least amount of harm to the experimental animals while still allowing us to conduct experiments in the context of the hematopoietic niche and relevant tumour microenvironment to ensure the relevance of the results to human health and disease. We are committed to conducting this research ethically and responsibly, ensuring the well-being of the animals involved.

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

Mice share a high degree of physiological and genetic similarity with humans compared to other less sentient organisms. This allows for a more accurate translation of research findings to potential human applications. There is a vast amount of research that has been conducted using mice, leading to the development of a wide range of well-characterized tools and techniques specifically designed for this model organism. This established infrastructure facilitates research and reduces the need to develop entirely new methodologies for a different species. Finally, the mouse immune system is complex and shares many similarities with the human immune system. This makes them a valuable tool for studying immune responses and developing immunotherapies for cancer.

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

We will regularly monitor the health and well-being of the mice throughout the experiment and establish humane euthanasia protocols based on predetermined humane endpoints. We will promote social interaction with littermates and using optimised environmental enrichment.

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

We will use guidance documents published by the NC3Rs (https://nc3rs.org.uk/), Institute for in vitro sciences (https://iivs.org/) and ARRIVE (https://le.ac.uk/dbs/animal-welfare/the-3rs/nc3rs-guidelines).

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

I will stay informed by signing up to and reading newsletters from NC3Rs, regularly attend workshops focused on 3Rs to stay updated on recent advances in the field of haematology and connect with colleagues to share best-practices and protocols. These advances will be implemented by reviewing and critically analysing experimental protocols throughout the project and consider if there are opportunities to further refine them based on new knowledge or emerging technologies.

10. Transcriptional regulation of immune cell function in inflammation.

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, transcription, neutrophils, macrophages, pro-resolution therapeutics

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 the project is to understand the mechanisms and pathways involved in controlling inflammation and the innate immune response. In particular, we aim to understand inflammation resolution and what transcriptional mechanisms contribute to the function of key inflammatory cells, in order to find therapeutic strategies for inflammatory diseases without impacting host defence.

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 plays a significant role in diseases like rheumatoid arthritis and Chronic Obstructive Pulmonary Disease (COPD). One type of white blood cell called neutrophils is heavily involved. Normally, these cells are short-lived, but during inflammation, they remain in the tissue at the wound site to fight off pathogens (e.g. bacteria) and help repair tissue. When inflammation is under control and the neutrophils have finished their job, the body usually gets rid of these extra neutrophils through a process called apoptosis

(controlled cell death) and clearance by other cells or migrate away from the wound site. However, in inflammatory diseases like arthritis and COPD, neutrophil function is dysregulated. They persist and cause irreversible damage by releasing harmful substances that damage tissue further.

Currently, we don’t have effective treatments to control this dysregulation of neutrophils. While neutrophils are important for fighting off disease, we need to find ways to fine tune the neutrophil response without compromising the body, leaving it susceptible to infection. Inflammation is a complicated process and needs further study to identify druggable targets for treatment.

We're trying to understand the mechanisms involved in the resolution of inflammation better so we can develop new treatments. Our goal is to find ways to regulate neutrophils more precisely to control inflammation and reduce damage to tissues. We're using advanced models in our lab to identify potential new drugs that could help resolve inflammation more effectively.

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

Work from our group has already given many insights into neutrophil function and has led to the identification of some promising ‘hit’ compounds that may have therapeutic value. This programme of work aims to build on these discoveries and identify targets and pathways involved in the mechanism of action of these 'hits'.

The short-term benefits will be to our research group and to other researchers in our field by generating more detailed models of inflammatory disease. There may also be short-term benefits to industry through the availability of new knowledge in drug targets. The data generated by this programme of work will be disseminated to the scientific community via publication in international peer-reviewed journals, as well as presentation at (inter)national conferences.

In the medium-term our programme of work may identify mechanistic pathways and genes involved in neutrophil dysregulation and lead further development that, in the long-term, may result in new ways of treating inflammatory diseases and more possible targets for therapeutic intervention.

In the longer term, these will lead to new therapies for patients that suffer from inflammatory disease.

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

Patients with inflammatory diseases suffer distressing symptoms, disability and death yet clinicians often have nothing to offer them in terms of disease modifying therapy. In large part this is due to a failure to understand inflammation resolution and what controls neutrophil function.

Short-term

Development of new models, approaches and knowledge will benefit the scientific community and may lead to advances in areas unanticipated at the start of this work.

Medium-long term

Patients with respiratory inflammatory disease will ultimately benefit, although it is our aim that we would use these therapeutics and knowledge to treat patients with multimorbidities that also involve inflammatory components not confined to the respiratory system.

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

We have extensive collaborations already established ranging from research groups at our own institute to research groups spread through the world. The areas of interest that these collaborations cover range from inflammation, infection, chemistry and human neutrophil biology, allowing a huge range of expert knowledge to be shared to achieve a common goal.

Findings are disseminated at national and international conferences, as well as being published in open access journals and in preprint services such as BioRxiv.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 23000

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 a vertebrate species with a comparable immune system to that of mammals. All major components of the innate immune system are present and share homology with their mammalian counterparts. Zebrafish are the optimal model for these studies. There are a huge range of transgenic reporter zebrafish lines readily available for visualisation of immune cell function, including cell death, cell migration, swarming behaviour, phagocytic ability. The transparent nature of the larvae makes them uniquely suitable for the visualisation of the cell biology of inflammation within a living host during the larval and juvenile stages. The regenerative capabilities of zebrafish allow further investigation into how altering the inflammatory response may impact tissue repair, a key process that needs to occur following an inflammatory response. Zebrafish are readily amenable to genetic modification, with gene knockdown and overexpression strategies easily achieved. Using larval and juvenile zebrafish stages are ideal as they only possess the innate immune system until around 3 weeks of age, allowing this part of immunity to be studied independently of the adaptive arm of immunity. We will also require adult zebrafish for breeding purposes to obtain embryos that will breed until 30 months of age.

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

The majority of fish experiments are performed on larvae before the onset of independent feeding – these larvae are not considered protected by the Animals (Scientific Procedures) Act.

During embryonic and larval stages, the zebrafish will be anaesthetised and fixed in agarose to enable imaging using fluorescence microscopy. Embryos or larvae may also be treated with novel compounds to investigate their effect on the inflammatory response or specific phenotype. Adults will be bred to obtain embryos.

In order to determine the outcome of manipulation of the inflammatory we will need to generate adult zebrafish bearing pro-inflammatory mutations or transgenes. This will lead to small numbers of animals potentially experiencing an exaggerated inflammatory response during their lifetime. These animals will not be subjected to further procedures, but will be observed and then killed by a Schedule 1 method prior to any suffering. We have not observed this theoretical phenotype in similar studies. These fish will be used for breeding until 30 months of age.

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

Most fish are expected to live out their lives in the same levels of comfort as any aquarium fish. A very small number may experience adverse effects such as oedema (mild swelling) e.g. around the heart and eyes. The majority of adults will be used for breeding only until 30 months of age without any detrimental health implications. Some adult fish will undergo the removal of a small portion of their tail fin for genetic analysis (fin clipping). Fish recover and regenerate their tail fin quickly. In a minority of cases (11% of total animals) fish will undergo tail fin transection when they are less than 5 days old and will be allowed to live until older larval stages or adulthood. Some gene mutations may result in unexpected adverse effects e.g. increased incidence of infection, however we expect this to be less than 5%. In these cases, fish will be killed without delay. Animals will be sacrificed before they suffer disease at the end of their natural lifespan, or earlier if indicated via Schedule 1 Methods.

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)?

Fish on protocol 1 breeding and maintenance >18 months - 90% subthreshold:10% mild.

Fish on protocol 2 generating transgenic and mutant lines – 100% of fish will be classed as mild. Fish on protocol 3 studying inflammation – 100% will be classed as moderate.

Fish on protocol 4 – 100% fish will be classed as 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?

Inflammation is a complex process involving many cell types, mediators and tissue environments. An in vivo model allows the study of how the components interact and affect the inflammatory outcome, something that can't easily be done in vitro using cell culture techniques. Using the zebrafish we are able to efficiently perform genetic manipulation that is not possible in any other system in which the innate immune system can be adequately

studied. Zebrafish are unique in the fact that have an isolated innate immune system from the adaptive immune system up to 3/4 weeks of age allowing precise interpretation of results without the complication of other immune pathways being involved.

Zebrafish also allow in vivo real-time imaging of immune cell behaviours that is not as easy to do or as invasive as in other models e.g. mammalian models. Where data are available in the public domain (eg. Bioinformatic and RNA seq data) or where studies have been done in other systems, these will not be repeated in our model.

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

Human cell culture – can use human primary neutrophils in vitro as well as neutrophil-like cell lines.

Ex-vivo models of inflammation are available for screening anti-inflammatory drugs. Identified through webofscience.com using search terms ‘non-animal models immunity’.

Bioinformatic analysis (in silico) – https://www.ebi.ac.uk/biostudies/arrayexpress. Can use published datasets to answer reserach questions.

Computer modelling - Can be used to investigate drug toxicity and binding potential in early stages of exploration. Used search terms ‘computer modelling as an alternative to animals models for inflammation’. Article in Frontiers 2018. Uses human data to predict drug toxicity.

Organs-on-a-chip – simplified human system with limited variables to screen treatments and test drugs. From article: https://pharmanewsintel.com/features/alternatives-to-animal-testing-models-in- clinical-and-biomedical-research

Why were they not suitable?

None of these are suitable, due to the requirements to model complex interactions between tissue environments and multiple cell types involved during the inflammatory response. Human cell culture and ex-vivo models do not allow pathways to be manipulated genetically or live visualisation of multiple cell types. Organs-on-a-chip is not suitable for the complex tissue and cellular interactions that occur during the inflammatory response in a whole 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?

Numbers of adult zebrafish required are calculated based on a requirement to have enough healthy adult fish at any one time to generate the larvae required for experimentation. Although large numbers of larvae can be obtained on one spawning, fish cannot then spawn for 2 weeks, meaning a rotating stock of fish is needed to ensure embryo supply. To minimise animal use, once experiments have been performed sperm will be frozen (for future IVF) and stocks will not be maintained.

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

We have performed pilot experiments in order to ascertain key timepoints. Exploratory experiments have been carried out where possible on larvae prior to independent feeding, to refine the hypothesis- driven experiments. We have searched on-line databases such as Array Express (https://www.ebi.ac.uk/biostudies/arrayexpress) to identify bioinformatic data which will allow us to narrow the range of hypotheses and sometimes to refute them without experimental 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 have sufficient pilot data to perform a priori power calculation to calculate group sizes. We will not initially exceed the predicted group sizes required to detect a 25% 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 25%. 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). Where possible adult fish stocks will be shared with existing PPLs, further reducing the number of stock fish required.

Zebrafish are highly fecund, so we are able to obtain large numbers of embryos <5.2dpf from a small number of parents. We will use pilot studies to optimise the number of animals used in this 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.

Zebrafish fin clipping and gamete expression methods rarely involve discomfort, but for all manipulative procedures animals are suitably anaesthetised (primarily for restraint). Anaesthetic is delivered by immersion (so no instrumentation is required) using an anaesthetic protocol suitable for the procedure and following best practice.

The methods for generating mutants and transgenics are optimised to minimise numbers and adverse effects are minimal. Generation of mutant/transgenic animals will be based on well established techniques for mutation and transgenesis, and the animals will not suffer directly due to the techniques.

Where feasible, early genotyping using a zebrafish embryonic genotyper (ZEG) system or 3dpf fin- clipping will reduce the number of larvae raised as we will only raise those of the required genotypes.

Our inflammation assays typically use read outs pre-5dpf, reducing the number of larval or adult fish required for our programme of work.

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

Zebrafish are the organism with the lowest neurophysiological sensitivity that is suitable to study the innate immune system and address our proposed aims. They have a number of advantages for these studies, including optical transparency, genetic tractability and extensive genomic resources.

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

For any experiments performed beyond the onset of independent feeding, larvae will be monitored regularly, with only the healthiest larvae being selected for inflammation studies, and individual experiments will be evaluated before further larvae are used in this protocol. The advantages and risks of this approach will be evaluated after each larva is used.

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

We will follow guidelines from https://science.rspca.org.uk on housing and care of zebrafish and published, well-established techniques for generating transgenic and mutant lines. We will use philosophies of experimental design advocated by the likes of Festing and Wurbel in order to refine our experiments.

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

I attend regular PI meetings where we discuss 3Rs and share ideas of how to promote 3Rs in our research. I subscribe to the NC3Rs newsletter to keep up to date with new developments. I will try to attend 3Rs presentations nationally. I also oversee the Zebrafish Seminar Series at our establishment to share best 3Rs practises.

11. Developing methods and tools for engineering the mouse genome

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Genome engineering, Validation and breeding, Conditional transgenesis, Reproducibility, 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?

We aim to develop, improve and quality control procedures for the more effective and efficient generation of genetically altered mice. This will deliver accurate robust and reproducible mouse models of human disease. For example:
We will quality control and validate transgenic reagents.

We will develop and establish advanced transgenic methods such as those employing RNA guided endonucleases and integrases to refine mouse models for the study of human diseases.

We will develop and establish new dosing regimens for the delivery of drugs that are used to activate enzymes that trigger rearrangements of gene sequences, thus changing the proteins expressed by the altered genes.

We will engineer genomes to express modified proteins and develop and establish in vivo systems for inducing the degradation of these modified proteins upon expression of relevant effectors proteins and/or delivery of corresponding activating drugs.

We will generate and/or check the quality of genome engineering reagents such as new embryonic stem cells for gene targeting.

Once the techniques are established and production parameters defined, the methods will be implemented under other project licences with the authority to generate mice of this kind.

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 is important as GA animals remain essential for biomedical research. New molecular biology tools developed in cell culture can also be applied to generate better mouse models: This includes models that contain sequences found in human, some of which are associated with ill- health. This also include being able to regulate more finely the expression of genes (at transcripts or protein level). Finally, it also means being able to modify gene functionality in a specific population of cells in the body or at a specific time, this allowing to explore cellular mechanisms. These models are increasingly complex, often involving many or large size changes in the genome, and require development of reliable protocols to ensure their reproducibility. Also, genome engineering techniques require a lot of animals and are currently broadly employed in an increasingly large spectrum of species. It is therefore very useful to improve methods for GA generation to make them more efficient so each project uses fewer animals.

Undertaking this work as part of a standardised genome engineering and breeding programme takes advantage of a large group with strong technical skills. It will result in the efficient use of animals.

Importantly, this programme offers the opportunity to reduce numbers for specific projects. It also allows for the introduction of new methods that produce refined more versatile or sophisticated engineered models.

Performing genome engineering projects under this licence allows us to optimise the use of the breeding colonies that generate the wild type (WT) animals for founder production. This ensures that engineering methods employed are optimised and consistent, therefore genetic alterations and backgrounds are validated, reproducible and describable for publication.

Quality controlling GA strains of mice ensures that subsequent experimental cohorts bred of these strains have a known, reproducible genetic alteration. The result of the experiments conducted on this licence and subsequent studies on the mice generated will be more reproducible scientific data as they are performed with controlled strains rather than non-standardised or uncontrolled backgrounds. Evolution of molecular biology techniques increases the depth of molecular validation needed which to date has revealed potential variability affecting the quality of new genetic alterations (e.g. untargeted genomic changes). Moreover, many new technologies modify embryos rather than cultured embryonic stem cells, as these technologies progress continual improvement in the depth of molecular validation of these new alleles is essential before they are used in experiments.

Genome engineering technology and the level of complexity of the engineered alleles are continuously evolving. The work planned on this project is important as it will yield new

protocols for the generation of refined designs for GA, using fewer animals and with better welfare.

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

The main output of the work will be improved methods to generate GA animals or reinforced quality checking procedures to characterise genetically altered mice.
Also, we will generate new genetically altered animal models designed after an in-depth assessment of the requirements for their use in downstream scientific projects, the existing published resources, and in line with the state of the art technologies in the field of animal modelling for biomedical research.

During this project we will quality check of genomic sequences or the consequences of introducing genome changes in GA strains.

A number of mouse lines produced on this PPL will themselves directly or via the data they produce lead to improvements of genome engineering processes.

The genome engineering work in this licence also yields a body of knowledge that is broadly applicable to the genome engineering field (including human) for improving both efficiency of genetic engineering and protocols to check the outcome of genetic manipulation. The dissemination of this information and expertise will be the object of regular method and commentary publications as well as inform the content of courses. It will also be presented at diverse relevant academic meetings (i.e. International Society of Transgenic Technologies or International Mammalian Genome Society) and online forum.

We will produce and/or check the quality of genome engineering reagents such as embryonic stem cell clones for gene targeting and new genome engineering reagents. This will include the development of new protocols that improve genome engineering efficiency or precision.

The work performed on this PPL also produces a body of information that will be used to inform risk assessment of clinical intervention that involve genome editing tools. This data will be readily shared with the clinical community.

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

The expected benefits of this project are new protocols and GA mouse lines of value for research in biomedical sciences.

The new protocols will serve to be more efficient, delivering better control of genetic changes in the mouse. This will lead to a reduction in the number of animals required for some protocols, allow better control of potential welfare impacts of founder animals and/or allow researchers to receive models more faithfully recapitulating human disease or more relevant to pertinent scientific questions. alleles.
In the short term this will lead to more reproducible experimental data. Longer term this will have lasting impacts for basic research, with the availability of thoroughly validated mice as models of aspects of human disease and pre-clinical models for drug development, as well as potentially reduce the number of mice required to produce genetic alterations.

New lines generated through peer reviewed programmes will be available every year to the global biomedical research community. We expect to deliver an estimated 20 new genetically engineered models a year during this project.

Deeper understanding of variability of genome editing outcomes which is a valuable knowledge to develop the clinical applications of genome editing.

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

The knowledge gained through constant re-evaluating of our genome engineering strategies feeds directly into training courses that we run several times a year on genome engineering, model validation and conditional transgenesis. Members of the teams involved in the genome engineering process also contribute to national and international specialist networks and have published advice on genome engineering and model validation processes. This team also regularly answer questions through dedicated mailing lists or receiving ad hoc queries from the community. In addition, we organise technical working groups with different facilities, where advances are discussed and disseminated.

This includes the sharing of negative results in order to prevent unsuccessful techniques being tried by other laboratories.

All new lines have an extensive molecular description, associated specific genotyping assays and welfare information is gathered. The archive is then annotated with any relevant information so it can be passed on to future researchers and the integrity of the strains can be maintained.

Members of this team, speak at conferences on current and future developments in genome engineering and validation techniques, focussing on the optimisation of the design of models for research as well as of the processes for model generation and for the quality assessment of these animals.

The team regularly publishes in scientific journals on process refinements as well as on their findings in terms of development of quality assessments of the genetics of the animals they produce. They also publish commentaries on the challenges associated with the use of genome engineering and genome editing technologies in particular in animal models and how that informs future application of the same technologies for therapies.

Species and numbers of animals expected to be used

- Mice: 52,850

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 licence to generate and validate improved protocols for the generation of genetically altered mice and new genome alteration designs. There is no need to age mice on this licence, but we will need mice at all other life stages for breeding and the techniques required for generating new GA lines.
Whilst this is not a service licence, there will be lines made on this licence that will go on to be used for other studies.

We aim to improve the process of alteration of genes associated with lethality or welfare issues in the mouse. This will involve the generation of mouse strains which have viability or welfare issues. In those cases, adverse effects are likely to be due to the gene alteration rather than the genome engineering techniques themselves.

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 molecular validation/genotyping and then they will be housed, either for transfer to another licence/project, kept as stock for potential future breeding, or bred with other genetically altered or wild type mice. A blood sample will be taken from a small number of mice for additional analysis. Any mice not moved on to other projects will be killed. Embryos may also be harvested for tissues after killing.

Mice may also be injected with hormones so they produce large number of ova. For generation of embryos these animals will also be mated. They will be killed shortly after these injections and embryos harvested for manipulations in vitro. A different cohort of females will be mated with sterile males to prepare them for pregnancy. Modified embryos will be implanted in foster mothers for the generation of founders of new genetically altered lines by either surgical or non-surgical techniques.

Inducing agents such as tamoxifen, doxycyclin, abscisic acid, gibberellin or auxin-like molecules will be administered to induce or repress activity of inducible transgenes.

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

The techniques used in this project have been designed to minimise suffering and animal numbers. The techniques are all frequently used and the likelihood of adverse effects is known to be low.

Making a new mouse model begins with producing embryos. Embryos are produced by injecting hormones into young females, this causes momentary discomfort.

Embryos are harvested post- mortem. After we have used technologies which cause the genetic changes in these embryos, they are put back into an expectant mother mouse by a surgical or a non-surgical procedure. The expectant mother is in a physiological state of pregnancy induced by methods such as mating to a sterile male and is therefore ready to carry a pregnancy. Under general anaesthetic, and with the administration of pain relief, embryos are surgically inserted into the reproductive tract. The surgery takes less than 30 minutes and mice will recover from anaesthetic within an hour. Mice are born around 19 days later.

After around 2-3 weeks a small ear clip is taken from the young, and in a small number of occasions a blood sample, is taken.

Where possible a non-surgical method to place embryos in the uterus of mice will be used in preference in all cases where sufficient robustness of the transfer method is demonstrated (blastocyst stage embryos).
When administering chemical agents we will select the mildest possible effective route of delivery: for example, if efficient oral gavage will be preferred to injection and addition of reagents to food will be preferred to oral gavage. It is possible that these agents themselves may cause adverse effects such as weight loss and reduction in activity.

During new and established dosing regimes the mice will be weighed daily, inspected carefully and dosing with discontinue if unexpected adverse effects are observed or the humane endpoints reached.

Some of the genome engineering projects aim at generating mouse models of human disease and as such may display some welfare issues associated with the condition they model. This might include developmental delay, behavioural and metabolic abnormalities. Each mouse model produced is monitored throughout its life for signs of ill health. Any welfare concerns are carefully monitored and the animals killed if they reach humane endpoint. Techniques used in this project have been designed to minimise suffering and therefore we aim to intervene at the earliest possible time. The techniques for generating new strains are all well used and the likelihood of adverse effects is known to be 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)?

90% of mice on this licence will have a severity of mild or below. 10% of mice may have a moderate severity as they undergo surgery, new dosing regimes or 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?

For the studies performed in this licence, it is not possible to use non-animal alternatives as in every case they need the complex interplay between ES cells and recipient embryos, the mouse genome in the context of early embryos and/or in multiple organ systems and only being apparent in a whole organism such as the mouse.

For example, when examining pluripotency and colonisation potential of ES cells after their microinjection and/or viral infection in embryos it is only possible to follow normal development for up to 4 days in vitro. The only way to determine the germ line transmission capacity of ES cells is to transplant microinjected embryos into a suitable recipient female mouse and breed chimeras born thereof.

Although it will not be possible to completely replace in vivo systems in the studies being performed, alternatives will be considered both prior to and during the experiments. Wherever possible, in vitro culture systems, such as analysis of cultured blastocysts rather than embryos transferred into pseudo pregnant females, will be preferred.

The very nature of the work we will perform under this PPL means there is no alternative to

using mice. This PPL was written to specifically cover the generation of new mouse models using microinjection technologies i.e. nucleic acid and injection or electroporation of genome engineering tools into the pronucleus/cytoplasm or injection of ES cells into a blastocoel cavity.

At the present time there is no reliable alternative to the use of microinjection or electroporation and embryo transfer techniques for generating new strains that allow detailed study of gene function in mice. As far as possible reagents used in the microinjection lab are quality controlled before being injected into an embryo.

Lower animal models such as fish are available but pathways to genome engineering are typically dissimilar to the mouse, as they require different methods of delivery of reagents and rely on different endogenous DNA repair machineries.

Members of the genome engineering team are active participants in working groups aimed at reviewing research models to identify and encourage implementation of replacement strategies.

Biological reagents (hormones, serum for cell culture) are frequently used in this type of work. As non- biologics including synthetic reagents become available, we will seek replacements of reagents from animal origin with materials from non-animal origin.

When new models are being made, to be used as part of another project, the necessity to use mice and the inadequacy of a non-animal method will have been reviewed through peer review of that project.

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

At this stage, non-animal alternatives to new lines are not considered as this project is dedicated to mouse genome engineering.

We will explore the potential use of non-animal replacement reagents in the genome engineering processes as they become available.

Why were they not suitable?

In most cases the techniques that will be transferred to mouse genome engineering will have been developed in vitro, 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?

Genetic modifications are delivered through the microinjection of chemical mixes containing genome engineering tools (enzymes or RNA) as well as DNA templates that

carry the information of the desired genetic modification. Alternatively, the genetic modification is introduced *in vitro* in cultured cells that are injected in early mouse embryos to generate animals that will found new genetically altered mouse lines.

Over many years we have gathered a huge dataset of all of our past efforts in generating new mouse strains. From this dataset we have analysed the likely numbers we will require for success. Typically in our current practices, 30-50 females will be used for embryo production for a microinjection session.

An additional 4 to 6 foster females will be used to receive the manipulated embryos.

From each of these sessions there will be 40-80 pups, all of which are potential founders. After molecular characterisation 2-4 founders bearing the desired modification, are mated to wild type animals, each of them producing 20-50 pups that are analysed for the transmission of the desired genetic alteration.

Different types of genetic modifications are generated with different efficiencies and the number of embryos used to attempt each project is selected according to previous experience with similar technologies/target genes.

In total this equates to 56 females + 50 potential founders + 35 progeny = 141 animals per session if they yield 3 positive founders, 106 if they do not. The team has a realistic capacity of 50 microinjection sessions/year for work on this licence over 5 years; Total number of animals over the course of the licence: 30,500.

In addition, a bank of 90 sterile males, renewed at regular intervals throughout the year is required for the production of foster females: this corresponds to 90*3 animals per year and a total number of animals over the course of the licence of 1,350.

Genome editing mouse strains which already carry a genetic alteration requires between 2-5 sessions of micro-injection, each using approximately 20-50 heterozygous females (each carrying only one copy of the allele to modify). This means that between 110 and 260 mice need to be per line, an average of 185 mice. Approximately 10 lines will be bred per year for this purpose. 9,250 over 5 years. We will aim to reduce these numbers by employing embryos generated with IVF from a heterozygous male. This however may not be efficient as only one allele in four represented in these embryos will be the one to edit (50% embryos wildtype, 50% embryos heterozygous).

Lines containing genome engineering tools such as integrase landing pads will require combining alleles to produce 20 to 50 animals and 20 to 50 embryos for expression characterisation (by analysis of reporter on fixed tissues or RNA extraction) or DNA recombinations (by genotyping assays). Approximately 10 allele combinations will be needed per year for this purpose corresponding to 5,000 animals over the course of 5 years.

Lines containing alleles for inducible protein degradation will require combining alleles to produce 20 to 50 animals and 20 to 50 embryos for expression characterisation (by analysis of fixed tissues or Western blotting). Approximately 10 allele combinations will be needed per year for this purpose corresponding to 5,000 animals over the course of 5 years.

Up to 50 females per year may be used for ovariectomy, corresponding to 250 animals, over the course of 5 years.

Overall, this equates to approximately 52,850 mice over 5 years, approximately 40% of these will be wild type.

This is an estimate based on current knowledge of future work, complex genetic alterations and different genetic backgrounds may change this significantly, therefore the total numbers of animals used in each experiment will be under constant review.

This is a very active research field where we do anticipate that the whole capacity of the team for protocol and model development will be utilised.

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

Many of the technical developments covered by this licence are themselves an effort at reducing numbers of animals used in transgenesis. At all times the minimum number of mice will be used to obtain a valid scientific result. This will be achieved by reviewing historical data associated with previous work carried out in this laboratory and others, or side-by-side protocol comparison to determine the number of mice necessary to produce the required number of embryos.

The number of superovulated females used for a microinjection session will be adapted if/when a higher success rate for founder production is anticipated and fewer embryos should be needed to produce the number of founders necessary for the experiment. Findings will be disseminated in publications and around technical networks, so improvements also benefit other genome engineering facilities. For example, the protocol for in vivo electroporation is effective for founder production with DNA donor up to 700 bases, and potentially represents a further opportunity for reduction of numbers of animals used for genome engineering if combined with AAV donors for larger knock-ins. We therefore propose to appraise the efficacy of the method combined with CRISPR/Cas9 reagents, as part of a small pilot study. Similarly, and in contrast to CRISPR-based genome editing tools, integrases produce highly predictable sequence integrations.

We will explore the use of these enzymes, including in combination with CRISPR/Cas9 reagents, with the aim to achieve enzymatically targeted integration of DNA segments in embryos with a predictable outcome. This will reduce the number of animals compared to other molecular methods that generate varied outcomes and where the offspring need to be intensely screened for the desirable new sequence with many carrying undesired genetic changes which are then killed.

We calculate individual superovulation and breeding strategies taking into account several factors. These include current embryo production and breeding data such as superovulation yield, viable embryo yield, litter size, mortality and fertility, from inbred lines, and where possible, on individual GA strains.

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

Excess embryos for genetic manipulation at one or two- cell stage can be frozen for use in a subsequent session.

New technologies will be embraced at the earliest possible time. For example, when more efficient in terms of yield, hyper ovulation and IVF will be used to increase the production

of one or two- cell embryos. However, the quality of embryos produced by this method need to be compatible with the required manipulation (for example, in vitro protocols can produce softer or more fragile embryos that are not well suited to pronuclear microinjection or may not survive well the stress of electroporation).

In cases where the likelihood of success of a genetic alteration is very variable between targets within a project type, a smaller pilot embryo manipulation will be carried out in the first instance to gauge efficiency and whether there are any unexpected welfare concerns in potential founders. A decision on a repeat attempt will be made according to the outcome of the initial session.

Many of the lines generated will be freely available to the scientific community shared between multiple scientific groups. Equally, groups of studs and some widely used transgenic lines (i.e. cre recombinase reporter) employed across many projects for many service users, avoiding the duplication of animal colonies.

Mice being generated through this project will be available through suitable repositories.

We will build on an initial small pilot for the establishment of primary culture of fibroblasts from biopsies to generate materials for structural or functional validation of new alleles so these can be performed without additional animals being bred for those purposes.

When testing new agents for inducing changes in the genome or changes in protein levels as a consequence of genome changes. we will run small controlled pilot studies initially.

These will assess the consequence or any adverse effects as well as efficacy and efficiency.

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 <5% of cases where a second biopsy is needed).

This should cause no more than slight and transient pain and have no healing difficulties.

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, most lines on this licence will be new, or bred in specific combinations for the first time. In these cases, it may not be possible to predict what the adverse effects will be. Humane endpoints will be put in place based on an extrapolation of the symptoms in

humans and predicted adverse effects in mice, using expertise based on the use of these types of models, from the user groups commissioning these new alleles. For example, where humans have a neurological disorder with movement abnormalities, we might expect tremors or gait abnormalities in mice.

We will prioritise methods where the production of homozygotes or trans-heterozygotes founders are inhibited specifically when targeting genes that are lethal or very deleterious or associated with severe phenotypes. This can be achieved by combining active and inactivated nucleases in the microinjection mixes so in most cells statistically at least one allele interacts and is protected by a non-catalytic enzyme, while the other allele may be targeted by an active molecule and mutated.

When we dose with chemical agents we will do this by the least invasive route for effective delivery, and repeat the lowest number of times needed to achieve efficacy of that agent. We will run small pilots, starting at lower doses for agents which are new to mouse or for which there is limited reporting of their use.

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

Embryos cultured to blastocysts stage are not reliable samples to determine outcome of genome engineering experiment as they represent small amount of materials that require large numbers of PCR cycles to generate signals which may need to artefacts. Furthermore, any DNA template delivered at one- or two-cell stage is likely to perdure in these samples and yield genotyping artefacts.

Many of the mice used on this licence have to be of an age that is suitable for breeding.

The very purpose of this licence is to improve protocols for mouse genome engineering and therefore requires the use of mice.

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

Foster females and potential founders born from embryo manipulations are followed with increased frequencies of welfare checks as new genetic modifications carry a higher risk of unpredictable welfare issues occurring.

We are now able to perform a non-surgical embryo transfer technique for our blastocyst work. Although this doesn't reduce the number of pseudo pregnant females used it allows us to use a less severe procedure. The females are anaesthetized for a very short time and recover quickly (no surgery to recover from).

We have introduced a gluture/suture technique into the transfer surgery. This is used to seal the initial back wound rather than clip. This removes the need to intervene after 7 days to remove the clip. Pain relief will be used routinely for surgery.

We have introduced a single use of needle policy for all injections. This has a large impact for this licence as it carries a large number of superovulation procedures.

CRISPR/Cas9 allows the generation of conditional alleles and lines carrying Cre recombinase thereby allowing some projects to circumvent viability or welfare issues encountered with a full knockout line. Whenever possible, we will prioritise methods where the production of homozygotes or trans- heterozygotes founders are inhibited when

targeting genes that are lethal or associated with severe phenotypes when both alleles are modified.

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

The establishment works under an accredited quality management system and under internationally recognised animal welfare standards. To conform to these standards we must ensure a high level of quality control on all aspects including husbandry and administrative processes.

Guiding principles for preparing for and undertaking aseptic surgery are taken from LASA guidelines.

For all 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 with sessions focussed on the 3R's in animal science such as the 3Rs symposiums, and the 3Rs session of the International Society for Transgenic Technologies (ISTT) 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 and other team members 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. In addition, managers working under this licence are active members of working group task with technology surveillance and development of use of alternative models.

The PPL holder is currently active within the ISTT and, in particular, it's 3Rs committee.

12. Gene therapies targeting the choroid plexus for the treatment of CNS 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

Hydrocephalus, Gene Therapy, CSF, Brain, neurodegeneration

Animal types	Life stages
Mice	adult, juvenile
Sheep	juvenile, adult
Pigs	juvenile, adult
Minipigs	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 evaluate gene therapies targeting the choroid plexus for the treatment of diseases associated with disruption to Central Spinal Fluid (CSF) homeostasis and physiology.

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 choroid plexus is a highly important structure for brain health and development.

Located within the lining wall of the ventricles of the brain, it produces and secretes the cerebral spinal fluid in which the central nervous system effectively floats, cushioning it from impact with the bony structures that surround it (i.e. the cranium and spinal column)

during movement. In addition, the choroid plexus also produces growth factors essential for brain development and peptides essential for tissue maintenance and healing.

Given the importance of the choroid Plexus in brain health and development, modulating the function of this structure would has potential benefits in the treatment of a number of clinically important conditions such as hydrocephalus, impaired developmental disorders, and neurodegenerative diseases.

The purpose of the outlined studies is to assess the feasibility of targeting specific cell subsets within the choroid plexus with gene therapies that either up-regulate or down regulate specific aspects of their function, with the long-term aim of providing improved treatment for a range of clinically important conditions.

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

The primary output from this project will be data on the efficacy and translational potential of gene therapies modulating choroid plexus function. This data will be disseminated via conference presentations and publications in peer reviewed scientific journals.

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

In the short term, the primary beneficiaries of these outputs will be the scientists and clinicians working on the project in terms of producing the data required to successfully progess the translate the work towards first in human trials.

In the medium term the work is expected to benefit scientists and clinicians working to find more effective treatments for developmental and neurodegenerative conditions of the brain by demonstrating the potential to deliver gene therapies to targeted cells within the choroid plexus.

In the long term, the work is expected to benefit patients and health care provider through the introduction of more effective treatments for a range of clinical conditions including hydrocephalus, neurodevelopmental disorders and neurodegenerative diseases.

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. The scientific team involved in these studies engage extensively in collaborative projects, which provide the opportunity to both gain expertise and disseminate knowledge.

Species and numbers of animals expected to be used

- Sheep: 36
- Pigs: 36
- Minipigs: 36
- Mice: 80

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 ensure the findings of the outlined studies are relevant to clinical conditions of humans requires the use of animal species that share a close anatomical and physiological similarity. Consequently, mammals will be used for these studies as they meet all the necessary scientific criteria and are well established as primary models for studying treatments intended for translation into humans.

Mice are the species with the lowest possible neurophysiological sensitivity that meets the required criteria for the studies. Wild type adult mice will be used for all primary proof of concept studies.

To fully assess the potential of therapies, shown to be effective in rodents, and to generate the translational data required to progress these into first in human clinical trials, studies will be undertaken using pigs, sheep or minipigs. Juvenile pigs will be used preferentially for studies of a short duration or for studies where the growth of the animal does not impact on sampling. Adult sheep or mini pigs will be used for studies where the growth of the animal could create technical problem for sampling, e.g. serial sampling of cerebral spinal fluid in longitudinal studies which require the indwelling sampling canula to remain precisely positioned for several weeks.

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

All animals: Following arrival at the unit, the animals will be acclimatised for at least 5 days before any procedures are undertaken. During this period, the animals will be habituated to humans by close contact and the hand feeding of treats. Large animals will also be habituated to the weighing crate and transport trolly by encouraging them to enter to gain food rewards.

Mice: A stereotactic surgical procedure will be performed under general anaesthesia during which a gene therapy will be injected into either the cisterna magna or a cerebral ventricle of the brain.

Following surgery, the mice are expected to make an uneventful recovery and to resume normal behaviour within a few hours. Upon recovery, the mice will be given pain killers, which will be maintained until they are showing no signs of pain. Mice will be maintained for a sufficient period for gene expression to occur (three to four weeks) and then killed, using a humane method to enable tissues to be collected for analysis.

Gene therapies, shown to be effective in mice studies, may be re-engineered and assessed for their translational potential using large animals.

Pig, mini-pigs and Sheep: On the day of surgery, the animals will be weighed and moved to the surgical suite using the transport trolly. Anaesthesia will be induced using an injectable agent, the animals will be intubated, and anaesthesia maintained using gaseous agents delivered by mechanical ventilation. The animal will be fitted with a stereotactic frame and imaged to determine the coordinates for implanting the delivery and sensory catheters. Up to three small holes will be made through the skull and catheters inserted to their target sites (cistern or ventricles) and secured to enable the delivery of therapeutic agents (eg: gene therapy, protein or physiological buffered solution such as artificial cerebral spinal fluid (CSF), the sampling of CSF and the monitoring of intracranial

pressure.

Following surgery, animals are expected to make an uneventful recovery and to resume normal behaviour within a few hours. All animals will be given pain killers upon recovery, which will be maintained until they are showing no signs of pain. At the time of surgery, or a subsequent time points, therapeutic agents (gene therapies) may be delivered, via the implanted canula, and/or samples of cerebral spinal fluid collected and cerebral spinal fluid pressure readings taken. These latter procedures are painless, and can be undertaken on a conscious animal without restraint by temporarily confining it in a small pen. In some instances (<25%) animals may have hydrocephalus induced, either before or after gene therapy, by introducing kaolin or blood into the ventricle of the brain via an implanted cannula. Animals undergoing this step will have their CSF pressure monitored carefully and if it exceeds a value indicative of hydrocephalus, or signs indicative of hydrocephaus occur, the animal will be killed using a humane method. At the end of the study period, all animals will be killed using a humane method, to enable tissues to be collected for analysis.

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

All animals: Transport stress: The delivery drivers are aware of the stress caused to animals during transportation and do their best to drive in a manner that avoids unduly stressing them however, it is not possible to completely eliminate this stress. Upon arrival in the unit the animals will be group housed in a clean cage or pen, with food and water, and left undisturbed for the first few hours.

Environmental stress: It is not possible to eliminate stress due to the change in surroundings however, stress will be minimised by providing the animal with an enriched environment that meet their needs, and not undertaking any procedures for at least 5 days following the animal's arrival on the unit.

Anaesthesia: Mice: Anaesthesia will be induced by exposure to gaseous agents within an induction box. The stress associated with this procedure is mild and usually lasts less than a minute.

Pigs: Anaesthesia will be induced by the intramuscular injection of an anaesthetic drug whilst the pig is confined within the transport trolly. Induction takes a couple of minute and is well tolerated by the animals which experience no more than mild transient pain caused by the insertion of a hypodermic needle.

Sheep: Anaesthesia will be induced by the intravenous injection of an anaesthetic drug. Induction by this route is rapid but requires the sheep to be restrained. Sheep tolerate physical restraint well and the insertion of the hypodermic needle causes no more than mild transient pain.

Surgery and imaging: All surgical and imaging procedures will be conducted under general anaesthesia and therefore do not incur any immediate suffering however, upon recovery animals that have undergone surgery are expected to experience some pain. To minimise this, all animals will be given pain killers, which will be maintained until the animal is showing no detectable signs of pain.

All surgical procedures carry a risk of blood loss and infection, these risks will be minimised by careful planning, fluid replacement therapy, and the use of a good aseptic

technique. The surgeons undertaking the procedure are all highly experienced in the techniques used consequently, complications due to blood loss or infections are not expected to occur.

Large animals only: Weighing and transport: To minimise the stress associated with these activities, the animals will be trained to voluntarily enter the weighing crate and transport trolly by giving them regular access to these devices and encouraging them to enter using food rewards.

Sampling: To undertake sampling the animal will be confined in a small pen. The sampling procedure is painless and is conducted while the animal is distracted by presenting it with food treats. Prior to sampling, the animals will be habituated to the sampling pen by encouraging then to enter to obtain food treats.

Killing: Animals will be killed under terminal anaesthesia and therefore will only experience the mild transient pain and stress associated with anaesthetic induction during this 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)?

Moderate 100% 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?

Whilst cell culture assays can and will be used to determine the ability of the gene therapy to target and induce changes in the cells of the Choroid Plexus, this technology does not enable the effectiveness of the intervention or its duration of action to be assessed. In addition, in order to gain approval for first in human trials, it is essential to generate safety and efficacy data using a large animal model.

Consequently, there is no alternative to the use of animals to address the aims of the outlined work.

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

Non-animal assay systems, using cultured cell lines and explant tissue, will be used to assess all new gene therapies. These technologies have already been successfully used by my group to demonstrate proof of principle for gene therapies targeting the Choroid Plexus.

Why were they not suitable?

Proof of principle studies for gene therapies targeting the Choroid Plexus have been successfully completed using cultured cell lines and explant tissue. However, it is not possible to assess the effectiveness of the intervention for the treatment of hydrocephalus or its duration of action using these technologies. In addition, in order to gain approval for first in human trials, it is essential to generate safety and efficacy data using a large animal model. Consequently, there is no alternative to the use of animals to address the aims of the outlined 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?

Care will be taken at each stage in the project to ensure that experiments are designed to use the smallest number of animals necessary to obtain the data required for statistical analysis. The study will follow a step wise design in which success at each stage will be required in order to justify progression to the next stage. We anticipate assessing up to 3 distinct gene therapies during the course of these studies. Following conformation of gene transduction in non-animal assays, therapies will be initially assessed in mice, comparing the therapy with its viral vector and focused on determining the effectiveness of transduction and its safety, in terms of tissue health and immune responses. Based on previous studies we expect a group size of 8 to be adequate to provide statistically significant results and therefore anticipate using up to 80 mice for these studies however, wherever possible, studies will be conducted in parallel to minimise the number of animals need for controls.

Therapies shown to be effective in rodents will be re-engineered for use in pigs or sheep. Studies in pigs and sheep will focus on demonstrating that the therapy is inducing the desire changes within the cerebral spinal fluid and will involve the collection of samples at predetermined time point. We anticipate assessing up to 3 gene therapies using the large animal models. Based on previous related studies we expect a group size of 6 to be adequate to provide statistically significant results and therefore anticipate using 36 pigs in short term studies and 36 sheep or minipigs in longer term studies.

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

Power calculations, using data generated during work conducted under my previous licence, were undertaken to determine experimental group sizes. Where appropriate, control group data obtained in previous studies will be used to minimise or eliminate the need for specific controls. Whenever it is feasible and appropriate to do so, studies will be run in parallel to enable a single control group to act as the control for multiple experimental groups. Experimental design software, such as the NC3Rs Experimental Design Assistant, will be used 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. Whenever feasible and appropriate, studies will be run in parallel to enable a single control group to act as the control for multiple 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.

Mice will be used for preliminary studies as they have the lowest neurophysiological sensitivity of the species suitable for the outlined work. In addition, the CRISPR-Cas9 platform that will be used to deliver the gene therapy is well established in this species, making it ideal from the perspective of treatment delivery.

Pigs, sheep or mini-pigs will be used for studies to determine the effectiveness and persistence of the treatments. The data generated in these studies will be used to support the translation of successful therapies towards the clinical setting.

The surgical expertise required to undertake the outlined work are well established within my research group for all species listed. Surgery will be performed, under general anaesthesia, using strict asepsis precautions. All animals are expected to recover uneventfully from surgery and to resume normal behaviour within a few hours. Upon recovery, all animals will be provided with post-operative pain relief which will be maintained until the animal is showing no detectable signs of pain.

The behavioural test used to assess the effectiveness of the treatment are not stressful and the animals engage readily in them.

The collection of serial samples of cerebral spinal fluid from large animals via an implanted catheter is painless and is well tolerated by the animals, which are readily distracted by feeding them treats. At the end of the study period, all animals will be killed using a humane method in order to obtain tissue for analysis.

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

In order for the studies to be relevant to the intended clinical setting it requires the use of animals that share a close anatomical and physiological similarity with humans. Only mammals meet these criteria.

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

The procedures used have already been refined during previous studies. Upon arrival in the unit, the animals will be acclimatised to their surroundings for at least 5 days. To minimise any stress associated with handling, during the acclimatisation period the animals will be habituated to humans by regular close contact and the feeding of treats.

Surgery will be performed under general anaesthesia by researchers with extensive prior experience and using strict asepsis precautions. All animals will be provided with post-operative pain relief, which will be maintained until the animals are showing no detectable signs of pain. The sampling and testing techniques used do not cause pain and are well-tolerated by the animals.

At the end of the study, the animals will be killed using a humane method.

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

All surgical procedure will be conducted in line with LASA guidelines.

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. I regularly review the NC3R websites which promotes the updated of new 3Rs initiatives. The AWERB committee at my institute 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 whenever they arise.

13. Identification and Pathogenesis of Microorganisms Associated with Bovine Respiratory Disease Complex (BRDC)

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

Livestock health, lung disease, microbiome, vaccines, antimicrobial resistance

Animal types	Life stages
Cattle	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 the project is to improve control of bovine respiratory disease complex through vaccination by i) providing an update on which are the most important pathogens associated with bovine respiratory complex in calves subject to normal farm vaccination protocols ii) begin the first steps in developing new vaccine candidates for *Pasteurella multocida* which is an important cause of BRDC but for which there is currently only a nasally delivered vaccine.

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?

Bovine respiratory disease complex (BRDC) is the most important cause of death and disease in the cattle industry despite the availability of vaccines against some of the most

important pathogens previously shown to be associated with BRDC. An up-to-date snapshot of the current situation is important to revise advice on control of BRDC. There is also the potential to identify pathogens not previously linked with BRDC. Understanding which are the most prevalent pathogens causing BRDC as well as assessing the presence of antimicrobial resistance (AMR) in these organisms is important for choosing the right treatment for affected animals and in addressing the problem of AMR. In addition this study will indicate whether new vaccine formulations are required. In addition we will identify targets for potential vaccine candidate antigens to protect from *P. multocida* infection.

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

This project will deliver new information about the pathogens that cause lung disease in calves despite current recommended vaccination regimens. This will inform veterinary treatment and may also identify gaps in immunity that could signify how to improve current vaccines. An additional output is information about the presence of antibiotic resistance genes present in the calf lung microbiome. The project will also investigate strains of P. multocida, a pathogen known to be associated with BRDC but for which there is currently only an intranasal vaccine for cattle. Identifying differences in membrane associated proteins in strains of high and low virulence and assessing their immunogenicity will identify key targets for vaccine development studies.

We expect that the information obtained will be the basis for at least 2 peer-reviewed scientific publications and articles in farming and veterinary publications.

In the longer term, the information could direct the development of new vaccines.

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

In the short term, the research team will benefit from using the information to underpin funding applications to continue work towards reducing the prevalence of respiratory disease in calves and cattle.

In the long term, understanding the changes in the lung microbiome associated with BRDC will be important in developing new or improved control measures for this common disease of cattle. The study could lead to improved advice regarding treatment of BRDC and/or better vaccines. Both would improve welfare of cattle and improve sustainability and profitability of rearing cattle. This is particularly relevant at a time when farmers are being encouraged to rear all dairy calves instead of culling dairy-sired male calves. (GB Dairy Calf strategy 2020-2023)

The information on relevant pathogens and antimicrobial resistance should lead to better targeted use of antimicrobials which ultimately has benefits for the human population as a whole, through preserving efficacy of available antimicrobials.

Further, control of farm animal diseases increases efficiency and reduces wastage in food production and thereby impacts the carbon footprint of farming and supports food security.

Relevant to improved vaccination strategies; P. *multocida* has been identified as an important pathogen associated with BRD, but there is currently no vaccine against it, except for an intranasal vaccine marketed by Merck. By identifying differences in membrane associated proteins in strains of P. *multocida* with different in vivo virulence, and assessing immune responses to those we will identify key targets for future vaccine development studies.

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

The results of this study will be informative and appropriate for publication even if we don't identify a change in pathogens associated with BRD compared to previous studies. That in itself would be an important finding.

If instead we do note new pathogens associated with BRD, new advice will be developed regarding recommended veterinary diagnosis and treatment. This will be disseminated to vets and farmers through various routes of knowledge exchange including articles in the farming or veterinary press, talks and/or webinars for farmers/vets and leaflets for distribution at agricultural events.

Identification of key P. *multocida* antigens associated with differences in virulence, and which are immunogenic, will provide further funding opportunities to investigate their protective efficacy in bovine challenge studies.

If we conclude that a change to current vaccines, or development of a new vaccine is required we would approach relevant commercial companies about taking this forward.

Species and numbers of animals expected to be used

- Cattle: Up to 62

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 type and age of animals will be used because this is the natural host and the age at which the disease problem occurs most commonly on farms and therefore will be most useful for this study towards developing better disease control.

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

Typically, calves will be brought in from a number of farms and housed together. This mixing of calves from different farms commonly induces BRDC naturally. The calves will be monitored and samples (nasal swab and blood samples) will be collected at regular intervals. If raised temperature or any other signs of disease are noted appropriate veterinary intervention will be used to return the calf to health, or the animal will be euthanised.

For the identification of key P. *multocida* antigens associated with virulence, calves will be infected with different strains that differ in their virulence. Animals will be monitored as for BRDC and blood samples will be collected at regular intervals for immunoblotting and mass spectroscopy to identify the major immunoreactive antigens.

What are the expected impacts and/or adverse effects for the animals during your project?
The animals in the naturally induced BRDC study and the P. *multocida* study are expected

to develop lung infections. In the case of the BRDC study, if clinical signs of disease are observed the animals will be euthanised as soon as possible and postmortem samples will be collected. For P. *multocida* infection, appropriate veterinary treatment will be given as soon as possible if clinical signs of disease are observed. Signs include raised temperature, subdued demeanour, inappetance, increased heart and respiratory rates. If such signs of disease appear the animal will assessed by a vet who typically will provide antibiotic treatment, together with non-steroidal anti-inflammatory drugs, which should resolve the disease signs within 24 hours. If clinical signs of disease continue the affected animal will be euthanised.
Venipuncture or nasal swab sampling will cause short term 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)?

In the naturally-induced BRDC study and the P. *multocida* study the severity will be moderate. Clinical signs of respiratory disease, considered moderate severity, are expected in up to 90% of the calves in the BRDC study and 100% in the P. *multocida* study. BRDC or P.*multocida* infection can develop quickly but any adverse effects will be of short duration because frequent monitoring will ensure rapid intervention when required.

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 other way to obtain the required information without using the natural host to mimic natural disease or to investigate the complex immunological processes and pathology that occur in response to infection.

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

We have previously used *ex vivo* precision cut tissue slices to investigate the effect of infection with pathogens associated with BRDC, including with P. *multocida*. However there is no non-animal alternative to gain the information required in this project.

We will use archived post mortem samples previously submitted for veterinary investigation to further our findings. However, these represent end stage microbiome and may not reflect the pathogens responsible during the active phase of BRDC.

Why were they not suitable?

Whilst we were able to get some information on innate immune effects of infection using precision cut lung slices they do not reproduce the complex immunological processes and pathology that occur in response to infection.

Obviously *ex vivo* cultures will not be informative on pathogens present as part of the natural lung microbiome of calves.

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?

20 calves should be sufficient to make sure we get a range of microbiome results. Previously the process of bringing in and mixing unvaccinated calves from multiple farms has unintentionally resulted in a high proportion of individuals developing BRDC. However, if very few calves become affected then we would need to repeat the naturally-induced protocol.

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

I asked for advice from scientists, vets and farm staff who have previously been involved in studies where calves have been brought here and housed together.

Although most of these studies were not regarding bovine respiratory disease it is clear that BRDC is a common occurrence in such animals although the number that will be affected is very variable.

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 run an intial study of naturally-induced BRDC and only if that does not generate sufficient BRDC cases (at least 8), we will run a second study. The animals that do not develop BRDC will serve as controls providing essential data on the microbiota of "healthy" lungs.

We will also use archived post mortem samples previously submitted for veterinary investigation but these represent end stage microbiome and may not reflect the pathogens responsible during the active phase of BRDC.

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 calves with naturally occurring lung infections or with induced P. *multocida* infection. The animals will be monitored at least twice daily for clinical signs of illness. If signs of disease are seen the animal will be assessed by a vet who will advise whether the animal should be euthanised straight away or whether appropriate veterinary treatment such as antibiotics and non-steroidal anti- inflammatory drugs should be administered. Although we expect that animals will become unwell we will limit the time-course as much as possible.

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

Only the natural host can answer the questions we propose about how to improve prevention and treatment of respiratory disease in calves.

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

The calves will be group housed to facilitate contact with other calves. This will allow them to have company whilst still making it easy to monitor individuals for milk/water intake, faeces output etc. The calves will be habitualised to human contact making it less stressful when they are handled for sample collection (nasal swabs and bloods) or for treatment if required.

Monitoring frequently (at least twice per day and at shorter intervals if necessary) will ensure that any signs of ill health can be noted early and the animals can be euthanised or veterinary treatment can be instigated rapidly for respiratory or any other type of disease.

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

Useful best practice guidance is found online at the 3Rs resource library https://www.nc3rs.org.uk/3rs-resources.

Care of the calves will also meet best practice for bedding, feeding etc as outlined in a number of publications.

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

The local 3Rs group is dedicated to keeping up to date on the latest advances in the 3Rs and to sharing any relevant information with staff. I have also found the 3Rs resource library on the NC3Rs website to be useful.

I am an active member of the Animal Welfare Research Network which is also a good source of new ideas to advance the 3Rs.

14. Neural control of puberty and fertility

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Fertility, Puberty, Neuroscience, Neuroendocrinology

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 understand how the brain is able to control the onset of puberty and how the brain controls fertility in adults.

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 studies in this project are expected to give us a better understanding of how key groups of cells in the brain function together to control the start of puberty and adult fertility. Many humans currently suffer from infertility or disorders related to puberty and we have little knowledge of how these important events are controlled. Approximately one in five couples in the UK will suffer from being unable to get pregnant and up to 1% of children fail to go through puberty at the correct time. The ability to correct fertility disorders from conditions such as polycystic ovary syndrome (PCOS) and speed up or slow down the pace of puberty in children with too early or delayed puberty would be very advantageous. The immediate benefits of this work will be increased understanding of how the brain works to control reproductive hormone secretion. Longer-term benefits will be those of providing new information that will allow for opportunities to make new treatments

for controlling puberty and fertility in humans.

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

This project will provide new information on the way in which the brain controls the activity of the gonads. It is aimed at understanding exactly how the brain controls the initiation of reproductive ability at puberty as well as how the brain controls the cyclical fertility of adult females. It is expected that these studies will provide the fundamental knowledge required to generate new treatments aimed at correcting disorders in the timing of puberty and adult human infertility conditions such as polycystic ovary syndrome and infertility due to stress.

This new information will be presented to the academic world at conferences and be available to the general public through lay presentations. It will also be provided through open-access publications in scientific journals. It is expected that this information will lead to better understanding of how the brain controls reproduction and this should enable better treatments to be developed for people with disorders of puberty and fertility.

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

In the short-term, results from this project will benefit other scientists within the UK and across the globe working on the same problem of understanding how the brain controls fertility and puberty.

We expect that understanding these mechanisms will also be very useful in developing new treatments aimed at helping people experiencing problems with puberty or fertility. For example, being able to speed up or slow down the pace of puberty in children with too early or delayed puberty would be very advantageous. Similarly, the ability to slow down the frequency of the pulse generator in women with polycystic ovary syndrome (PCOS) would be expected to help tremendously with their sub fertility.

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

The impact of this work will be maximized through - presentation of findings at scientific conferences and lay user-group meetings publication of all work (positive and negative observations) in open-access journals providing all source (original) data alongside each publication to enable re-use and re-analysis by other scientists.

Species and numbers of animals expected to be used

- Mice: 9,120

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.

At present, the only way of investigating how specific groups of brain cells interact with one another to control processes such as puberty and fertility are through genetically-altered animal models. The great majority of these models have been developed in mice and enable the activity of specific cell populations to be modified or monitored whilst

determining the effects on reproductive hormone secretion. Our work examines how fertility is initiated at puberty and then controlled in adult mice.

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

The great majority of the animals used in this project will be used for breeding and experience no adverse effects.

A typical recording experiment will involve an animal being anaesthetised for 1-2 hours and a very thin recording probe (less than 0.5 mm wide) inserted into a specific brain region with tiny screws placed into the skull and everything secured in position with dental cement. Animals may experience some discomfort and pain after surgery and will be treated with analgesics before and after surgery. After surgery, animals will be accustomed over several weeks to having their head probe connected to a very thin cable so that recordings can be made while the animals are free to behave normally in their cages.

These recordings can occur for a few hours up to a maximum of 5 days so that we can record the activity of cells across the entire estrous (reproductive) cycle of a female mouse. The estrous cycles of mice are determined by looking at the cells from the vagina that are obtained by gentle water flushing. A few of these animals will, in addition, experience mild and transient discomfort from blood sampling during the recording sessions in which very small blood samples are taken from the tail of the mouse. Another small group of animals will be put on weight loss diets that are not expected to cause distress but result in weight loss up to 15% of their normal body weight. The final procedure involving perfusion fixation (the administration of a solution to embalm the mouse) will be undertaken under non- recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and may experience mild distress and no pain.

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

Animals will have surgery to implant small recording devices into the brain so that the activity of cells can be recorded. They are expected to recover quickly and will be given painkillers for two days following the surgery and post-operative care just like people recovering in hospital. Very occasionally, there can be a problem with the recording devices and an animal may need a brief further operation to correct this. In some cases, animals need to be placed back into their cages by themselves after the surgery to prevent other mice from tampering with the head devices. In these cases, mice are kept for the rest of the experiment in "open top" cages beside one another so that they can hear, see, and smell each other.

The blood sampling takes tiny amounts of blood that, together, never exceed more than 10% of an animals total blood volume. This is the same as a human giving a blood donation.

Animals will receive weight loss diets for up to four weeks that will reduce their weight by up to 15% before being placed back on normal food.

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: 86% mild, 14% 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?

At present, the only way of investigating how specific groups of brain cells interact with one another to control processes such as puberty and fertility is through genetically-altered (GA) animal models.

These processes are so complex that currently they can only be examined in animals. No artificial system is able to replicate this at the moment. The great majority of GA models have been developed in mice and enable the activity of specific cell populations to be modified or monitored whilst determining the effects on reproductive hormone secretion.

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

Mathematical modelling. This is where people use mathematics to try and generate realistic models of how the brain functions.

Cell cultures including new preparations called organoids where parts of body organs are grown in laboratory dishes.

Why were they not suitable?

We are actively engaged in developing mathematical models for understanding the neural control of fertility. However, these models are severely limited by the lack of fundamental understanding of the cell groups involved in the process. As we generate more fundamental data this is being used to improve the quality of the mathematical models.

A cell culture approach for investigating fertility was developed in the 1990s (GT1 cells) but failed to provide any insight into the way individual brain cells work together to control reproductive hormone secretion.

Organoids are increasingly being used to model complex structures. However, at present no organoid preparation exists for the hypothalamus, the brain region where the cells controlling puberty and fertility reside.

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 animal numbers based as far as possible on projected experiments over the next 5 years and also taken into account the numbers of animals used in the current 5-year project license that uses very similar methodologies and approaches.

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

To achieve good experimental design, we have established clear experimental objectives and used randomised controls in which, as far as possible, the primary investigator is blind to the experimental groupings. The use of inbred rodents in a consistent cage environment with skilled experimenters further minimizes variability.

Our studies use “Fully Randomised” and “Repeated Measure” experimental designs as appropriate to minimize animal numbers. Fully randomised means that all variables are treated equally and treatments are given without any bias. A repeated measure design means that a series of measurements are made from the same animal so that, for example, the control treatment and test treatments are performed in the same mouse. This provides a more robust ability to detect changes to any treatment and uses very many fewer animals compared to separate groups of mice being used for each measurement or test.

The animal sample size for our work is typically 6 animals per group. This is a well-accepted sample size in our field and provides sufficient robustness for statistical analysis. However, we undertake Power Analyses for each experimental treatment and adjust the required animal number as required. Much of our work requires the placement or injection of tiny recording devices into very small brain regions. Past experience shows that even the best surgeon only has an average 75% success rate for the brain regions we target. It has been my practice that anyone in the laboratory unable to achieve over 50% success after sufficient tutoring is removed from these types of studies. Thus, for all experiments of this nature we will prepare 9 mice in the expectation of being able to use 6 mice in experiments. In circumstances where we end up with more than 6 surgically prepared mice, all will be used for experiments.

Experiments will be designed so they can be published in accordance with the ARRIVE guidelines. By archiving tissue for new studies and developing recording devices that signal information without restraint, animal welfare can be improved, and data collection can be maximised while minimising the number of animals used experimentally.

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

The breeding of GA rodents is managed and coordinated across the research group by a very experienced Laboratory Manager in consultation with the principal investigator. This occurs in accord with the NC3R breeding and colony management guidelines and ensures the efficient management of the various lines and that sufficient animals are produced in a timely manner for the 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 use highly refined methodologies that allow the activity of brain cells to be monitored and changed in unstressed animals. We do this by allowing the animals to become very accustomed to the recording environment as well as their individual investigator over several weeks. This is very important for us as any stress stops the activity of the cells in the brain controlling fertility that we are trying to investigate.

Aside from the surgery itself, where animals are treated like humans in hospitals, the mice experience no pain or suffering over the course of the recording experiments. We use a highly refined method of taking multiple blood samples from mice in which a tiny (less than 1mm) portion of the tail tip is removed to allow frequent blood samples to be taken over 1 to 4 hours without stressing the mice. This procedure was co-developed by our group and is now used throughout the world as the best practice for examining pulses of hormone secretion in mice.

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

The brain mechanisms controlling fertility appear to be very similar across all mammals so that use of mice is appropriate for understanding function relevant to humans. The way in which and the brain of an animal like a fish controls reproduction is quite different to that of mice and humans.

We are trying to understand how the brain controls fertility in the dynamic real-life situation. Any anaesthetic stops these mechanisms from occurring.

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

We are continually looking to refine experiments through observation and the trialing of small changes to our procedures. For example, this has led to our present ability to house some animals together after brain surgery operations. Animals are housed together wherever possible in cages that have environmental enrichment such as tunnels. In the past, it was considered dangerous to house animals together after they had received head surgery as the mice would interfere with the head implants.

All surgery is undertaken using aseptic procedures and mice receive regular pain medications following surgery and are monitored for any post-surgical bleeding or weight loss. Pain relief following surgery is provided through food the animals eat. Once mice have recovered, they are accustomed to the experimental recording environment and the human investigator by gentle handling at least five days every week. This can continue for several months so that the mice become very familiar with the scientist.
The tail-tip bleeding procedure is performed after extensive habituation of mice with the same investigator handling the mouse and stroking its tail every weekday for several

weeks. We have trialed applying local anaesthetic to the tail tip before cutting and bleeding but it is difficult to determine if this has any impact as the mice do not react to cutting the tip of the tail in any case.

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

We use the ARRIVE and Norwegian PREPARE guidelines, and take advice from NC3R on the management of breeding genetic mouse lines.

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

This occurs internally through information provided by the local AWERB, NACWO and NIO and externally, through local and international scientific meetings. In particular, the UK National Centre for the 3Rs (NC3Rs) provides newsletters to help keep up to date while the Laboratory Animal Science Association (LASA), Institute of Animal Technology (IAT), and the Royal Society for the Prevention of Cruelty to Animals (RSPCA) all provide additional sources.

15. Brain and behavioural mechanisms of memory in health and in dementia

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Memory, Dementia, Aging, Anxiety, Behaviour

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	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 characterise brain regions and behavioural factors involved in learning and memory. To understand how cognitive and mental functions are affected in aging and in neuropathological conditions, such as dementia. To use the knowledge for developing methods to improve cognitive wellbeing across lifespan.

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 is a major global challenge. The number of people living with dementia is estimated at around 850,000 in the UK and more than 55 million worldwide. Common symptoms in dementia include memory loss, difficulty in memory retention, and deterioration in cognitive abilities. Additionally, a proportion of people living with dementia also show mood and emotion changes, including signs of anxiety. As a consequence, independence in living and life quality of people with dementia and their families are significantly affected. One of the main factors that contributes to dementia is aging, while advanced aging is also associated with decline in cognition in later life. Therefore, it is

important to understand what cognitive or emotional processes underlie the symptoms in models of dementia and in aging, what behavioural factors can slow symptom decline, what cellular and circuit changes in the brain are associated with the symptoms. The ultimate goal is to use the knowledge gained to develop behavioural, pharmacological, and circuit interventions to maintain cognitive wellbeing over time.

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

The main output from this project will be the development of models to characterise cognitive processes that are affected in aging and in dementia. It will inform what procedures or pharmacological targets in early life can be introduced to improve learning and memory in aging and to delay symptom onset in dementia. It will provide knowledge on brain regions or circuits in mediating functions that are affected by aging and dementia. These will be communicated through publication and scientific and public engagement.

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

The researchers and trainees involved in the project will gain research skills and provide discovery. Undergraduate and postgraduate will benefit from courses covering the current state of the knowledge and be inspired in their career choices.

The scientific community will benefit from novel insights and models that this project provides. This will include scientists in neuroscience, geriatric, and dementia research. The main pathway to reach the academic community is through seminars, journal publication, and conference presentation.

Charitable organisations with missions in improving healthy aging or curing dementia can also benefit from knowledge developed in this project to inform possible novel therapeutics. Industries in preclinical and biological research can also benefit from the research activities and findings that inform new designs of materials and devices. People living with dementia may benefit from the outputs through future translational research with clinical trials and improved guidelines.

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

To maximise the outputs, highlights of out publication can be arranged through the publisher and through the press release office of the establishment. Presentations in national and international events will be sought. Examples are national and international Neuroscience Associations.

We will also interact with charitable organisations with missions in improving life qualities in aging or in curing Alzheimer’s disease via presentation at their network meeting and international conference. We will interact with industries in preclinical and biological research to ensure best designs in research can be developed and implemented.

Species and numbers of animals expected to be used

- Mice: 3500
- 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.

In order to determine cognitive, behavioural, and physiological changes due to dementia pathology, genetically modified rodents carrying risk genes that contribute to brain pathology will be used to model Alzheimer's disease. Because brain pathology can be detected from young to middle adulthood so most of the research will aim to describe changes in the early phase of the disease. Because aging is an important risk factor in leading to physiological changes, it it vital to also determine what and how cognitive functions decline during the aging process. Because cognitive processes plays an important role in anxiety in health and in dementia, young adult rodents will be needed for modeling aspects of neuropsychological symptoms and for developing therapeutics.

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

Animals may be bred from lines carrying risk genes associated with Alzheimer's disease (typically subthreshold). Some animals will receive basic behavioural assays to determine the phenotypes of emotion, motor, and motivation (typically mild). Some animals will receive more advanced behavioural assays with spatial tasks to characterise their learning ability and memory retention (typically mild, some moderate). To determine therapeutic potentials and biological mechanism, they may receive chemicals through injections before or around the time of behavioural assays. To understand the brain mechanisms that contribute to the cognitive function or therapeutic effect, a smaller proportion of animals may receive infusions and probes to label, to stimulate, or to inhibit focal brain regions (some mild, typically moderately).

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

Most animals will engage behavioural exploration that does not cause adverse effects. Assays for aversive learning and memory can cause transient discomfort during brief procedures in the conditioning chamber, which would not affect their wellbeing in the home cages. Brief footshock delivery can cause transient pain that subsides within seconds. Application of certain chemicals may cause short-lasting motor changes that recover in a few hours. Food restriction can cause body weight loss during the phase of food restriction, but body weight recovers rapidly when food is given ad libitum. For aging research, a small portion of aged animals may develop aging-related diseases or symptoms, such as tumours, as part of the aging process. Aged animals will receive regular checks for wellbeing. Brain surgery for focal infusions or implantation can cause initial discomfort which will be mitigated by anesthesia during the procedure and analgesia during and post procedure to prevent or alleviate 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)?

It is expected that the severity experienced by approximately 44% animals to be sub-threshold, approximately 22% animals to be mild, and approximately 34% animal 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 aim to understand the causal role of brain circuits and molecular mechanisms in learning and memory. Using rodent behavioural models allow us to investigate leaning and memory that are previously established to mimic human cognition and to apply genetic, pharmacological, or surgery tools to build causality. Also due to a high degree of genetic similarities and brain structure similarity between humans and rodents, rodents will be need in this project to achieve the aims.

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

Tissue electrophysiology may inform how stimuli lead to physiological changes. Cell recordings may inform how cells respond to stimuli. These are often derived from animals tissues although can possibly be obtained from post-mortem human tissues or from human cells. Mathematical modelling can simulate aspects of computational processing seen in humans.

Why were they not suitable?

Tissue electrophysiology or cell recordings will not reflect how an intact organism performs learning and memory. The targets identified from cells or tissues cannot capture the full complexity from an intact system with various circuit inputs and outputs. Mathematical modelling cannot replace the animal research that provides a holistic consideration on genetics, environments, neurotransmissions, neuromodulation, and circuits.

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 estimation is based on previous research in the field and from our work. The procedures will be refined and within-subject designs will be used whenever suitable to enable reduction. The effect sizes from our previous studies in wildtype animals is very high with male-only research. With an emphasis on research with both males and females, the number will increase accordingly based on our recent work. To factor in the dementia research with genetic models of Alzheimer’s disease, the numbers of animals that are requested in the previous Home Office approved PPL are also referred to and the breeding schemes to achieve sufficient animals carrying risk genes are computed. Parts of the research will require aging animals and hence age associated attrition is considered.

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

Within-subject designs will be used when appropriate to reduce the amount of animals needed. The experimental design is based on the current guidelines of good practice. Statistical power analysis is performed to plan studies. Multi-factorial designs will used to increase statistical power and reduce the number of animals required. Wherever possible we will take multiple measurements in the same animal over time and across age stages to maximise the information per animals which will reduce the number of animals needed overall.

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

The experimental devices, such as event arenas or conditioning chambers, will be carefully designed and calibrated to keep the consistency in hardware. The environmental factors such as temperature and noise level will be monitored to reduce variation and the number of animals needed. The experimenters will be properly trained and assessed for good animal handling. Animal handling will be properly implemented to reduce the stress which will reduce data variation and the number of animals required. When appropriate, animals will be habituated to experimental devices and the basic procedures to increase the quality of subsequent data collection which will also contribute to fewer animals being 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.

To model neurodegeneration symptoms, rodents carrying genes associated with Alzheimer’s disease (AD) pathology or inflammation will be used. To visualise brain cells, mice carrying fluorescence proteins will be used. To activate or inhibit brain cells, mice with genetically altered proteins will be used. The AD mice may cross with the later 2 to enable visualisation or manipulation of the brain cells. To understand learning and memory, wildtype animals and GA animals mentioned above will be used. No pain or harm is expected with these animals.

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

Cognitive functions (e.g. spatial learning and memory, object recognition, cue-consequences associations) in this project cannot currently be captured by animals that are less sentient than rodents, such as worms. When investigating memory functions that require prior knowledge, rats may be used as their performance can be more consistent and less variable (hence less animals will be used). Genetic models for dementia are better developed in rodents than in less sentient animals. The brain structure in rodents, as opposed to animals that are less sentient, is also closer to that of human brains.

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

We will adopt refinements as described below to increasing experimental quality and optimise animal wellfare.

Animals will be group-housed at breeding and study outset. Cages will be enriched with tubes, paper domes, wooden bars, and cotton pads for exploring, chewing, or nesting to reduce stress and promote social interaction. Surgery will be performed under general anesthesia, using aseptic technique. Pain relief will be administered during recovery to minimise distress.

Staff will be trained, assessed, and equipped with knowledge on regulated procedures and are aware of signs of animal wellbeing. The facility is maintained with specific pathogen-free status. they will be carefully monitored to ensure that suffering greater than minor and transient in their home environment does not occur.

Body weight will be monitored during food restriction to ensure stable weight maintenance. Animals will be carefully monitored after delivery of aversive stimuli, such as mild, brief footshocks, or water exposure. The procedures are refined based on our or others' publications on relevant research questions. Aging animals will be carefully, and regularly monitored with carefully designed forms to ensure their wellbeings as they reach the aging criteria.

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

We will follow local updates on best practice guidance provided by our institution. We will also follow procedures that we and others have published with refined methods for specific experimental questions we aim to address in this programme. We will seek updates on best practice guidelines published in the NC3Rs website (www.nc3rs.org.uk) and from ARRIVE guidelines.

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

We are in regular contact with the NACWO and NVS to review current approaches and whether there are any new 3Rs opportunities. We subscribe to the NC3Rs e-newsletter to identify 3Rs developments that are relevant to our protocols. We attend local user meetings for advice and guidance around good practice and use of 3Rs. We will check use the following guides and databases on alternative approaches (e.g. The EURL ECVAM Search Guide for alternative strategies and methods to animal- based research. FRAME on the basic principles of searching for 3Rs information. ORECOPA on alternatives to the use of animals and information about implementing the 3Rs). We will also regularly review databases specific to our programme of work.

16. Developing Multispecific Antibodies For Cancer Therapies

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, Immuno-oncology, Antibodies, 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?

The aim of the proposed programme of work described in this document is to discover and enable preclinical development for potential new antibody therapies for cancer using our clinically proven bi- specific antibody platform. In this project, we aim to generate in vivo drug efficacy and pharmacology data (including Pharmacokinetics (PK), pharmacodynamics (PD) and mechanism of action data) to support the selection of new therapeutic candidates to ultimately advance to Investigational New Drug (IND) filing (or equivalent) and into 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?

The International Agency for Research on Cancer (part of the World Health Organisation) reported that, in 2020, there were 19 million new cases of cancer and 9 million cancer-related deaths worldwide.

Despite the advances in cancer therapies and the reduction in the number of deaths from previous years, the number of diagnosed cancer cases is increasing due to people living longer, population growth and cancers being identified earlier, therefore cancer is still one of the leading causes of death worldwide and there is still a need to find more efficacious therapies.

Our goal is to continue to deliver life-changing treatments to cancer patients and other serious diseases using our technology to lead to development of new therapies using our mAb²™, a novel class of bispecific antibody-based therapy. Natural antibodies only recognise a single target. Our technology provides a platform to generate bispecific, trispecific or multispecific antibodies that can recognise two or more targets.

These antibodies can be conjugated to small molecules called payloads with specific functions, e.g. cell-killing cytotoxic drugs or immune-stimulatory agents, to generate a new class of therapeutics called Antibody-Drug Conjugates (ADC).

Our continued development to understand how to apply our technology to modulate the complex interplay between the tumour and immune system, means we have been able to assess several potential therapies and will be identifying opportunities for where these can be deployed on new projects and explore and bring an immunological answer to complex tumour physiology which is not possible to attain with combination approaches.

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

The main output of this work will be a greater scientific understanding of the way our new therapies may work in vivo (in a living organism)

- This will include pharmacokinetic parameters (how much and how long the therapy is present in the organism).
- How to target these molecules to a particular cell or organ, how to dampen immune responses, or how best to control the activity of these molecules in vivo.
- In vivo efficacy to alter disease pathology and progression.

This will inform specific drug projects that are aiming to produce new medicines for patients, and where applicable this work will be published in scientific journals, presented at conferences, or used in patent applications.

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

The benefits of the proposed work under this project licence are as follows:

- Development of new medicines for patients to increase the overall survival of patients with advanced cancer.
- Our multi-specific antibodies may offer a more targeted therapy that improves the therapeutic window providing treatments with fewer side effects.
- Multi-specific antibodies have the potential to reduce the number of different therapies a patient must receive.
- Contribute to the wider scientific community to enhance understanding of tumour immunology and drug discovery processes.

From the generation of multi specific antibodies to the clinic and testing in cancer patients, a systematic approach is taken to select molecules with the best chances of benefiting patients by generating data packages that confirm the proof of concept, translatable pharmacology and supporting the understanding of mechanism and biology.

Demonstrating improved efficacy of these bispecific products which have already demonstrated success in the clinic as monoclonal antibodies will be immediately translatable.

Treatment of human cancers with novel “2-in-1” bispecific and new exploration into multi-specific antibodies will have an immediate impact on human health and support the lead candidate selection of bispecific and multi specific antibodies against novel targets. Our data packages will support the translational process by providing guidance on the development of biomarkers for the clinic and selection of the most appropriate patient population. Bispecific antibodies developed under our previous project licences are now in clinical trials to evaluate activity in patients with advanced cancer.

Expected deliverables for data generated under this project proposal are:

- Select 1 new candidate per year and 2-3 Investigational New Drug (INDs) over the course of this project.
- Generate patent filings to document new inventions. This protects research findings and enables data to be brought into the public domain to share with the wider scientific community.
- Share knowledge with the wider scientific community via publications, posters, conference talks and patent submissions. Talks and presentations of data at least 5 conferences per year.
- Publication output is anticipated to be about 1-2 publications per year.

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

As with previous project licences, we are always keen to share our learnings with wider scientific community via publications. Also, wherever applicable, the company will engage in scientific collaboration and contribute our knowledge gained in tumour immunology and drug discovery.

Our work is a collaboration of multiple matrix teams, working on various aspects to develop the multi- specific antibodies. We strive to synchronise multiple studies to maximise the information obtained and use from each animal in our research, e.g. collecting different tissues and samples for multiple studies from the same animals.

We also create online data resources to enable researchers across matrix teams within the company to access and mine data based on the individual teams’ interests from multiple angles to maximise the findings from the use and data extracted from the same animals.

Species and numbers of animals expected to be used

- Mice: 17,500

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.

Murine models have been shown to yield reproducible results in previous studies carried out under our project licences and in other laboratories with respect to the control of tumour growth by the immune system.

The comparison of mouse and human immune systems shows strong parallels between cell types and functions, in both health and disease, although some details vary across the species.

Adult mice usually 8-14 weeks of age are chosen because their immune system closely resembles that of humans, inbred strains are available, they are relatively small, and easily genetically manipulated.

Therefore, they are currently the best non-primate model for human immunology research.

The current accepted research for drug preclinical development is strongly weighted to match previous data. Mice meet, and are, the standard used by regulators for preclinical development of drugs and their requirements for initiating clinical trials.

For any other mammalian species, the level of basic immunology knowledge is much lower, and an enormous amount of validation work would be needed before the project could be initiated.

Furthermore, many tools are already developed for mouse work, including optimised immunomodulatory drugs and processes for genetic modification.

Mouse models will only be used where there is a rationale that the desired mechanism is relevant in the murine system, and where no suitable in vitro systems can be used as an alternative.

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

Animals will be housed in Individually Ventilated Cages in groups of 3-5 animals with access to food and water Ad Libitum. Each cage will have environmental enrichment provided (including multiple types of nesting/bedding material, cardboard tunnels, foraging seeds and wood chew blocks).

Animals on a study may be microchipped or tail marked with a marker pen for the purpose of identification and animals will receive at least 1 week's acclimatisation period prior to starting the study. Animals may be randomised into groups, and we expect the mice to remain in these group sizes without incident.

At any time and as needed, substances (control or treatment, e.g. antibody) may be administered at the volumes and frequencies not exceeding the limits within the licence using standard routes (intravenous, subcutaneous, intraperitoneal or by oral gavage).

Administration of substances may be given whilst the animals are restrained by ‘scruffing’ or restrained in a red transparent plastic tube during the procedure.

Whilst undergoing regulated procedures, we expect the animal to feel no more than mild, transient pain and no lasting harm, as the least invasive route, volumes and frequencies for administration or sampling will always be chosen.

For subcutaneous tumour-bearing studies, the animals will have tumour cells implanted into the flank and tumours will be allowed to grow with routine monitoring up to the published guidance limits. For an animal carrying a single tumour, the mean diameter should not normally exceed 1.2 cm in growth studies and will not exceed 1.5 cm for therapeutic studies (Workman et al., 2010).

After implantation of the tumour cells, daily welfare checks and clinical observations to measure tumour volume and body weight will take place twice a week when the tumour becomes palpable.

Once tumours reach a measurement of 10x10mm3, the clinical observations will increase to a minimum of 3 times per week. Animals with tumours likely to exceed the limits of the licence based on the last measurement and the expected growth (additional considerations will be made for weekends) calculated using previous data points, will be humanely killed by an appropriate schedule one method and tissues collected as required for analysis.

Blood samples will be taken within the volumes and frequencies of the published guidance to minimise the discomfort experienced by the animal from either the blood sampling procedure or from the restraint during the procedure.

Animals showing body weight loss will be supplied with nutrient gels and given daily clinical observations until weight improves, If there is no improvement or animals show a weight loss of 15% they will be humanely sacrificed.

Terminal procedures will be performed under non-recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and therefore may experience mild distress and no pain.

At the end of a study, animals will be humanely killed and tissues taken for ex vivo (outside of the living body) analysis.

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

Animals in our studies will be inoculated with tumour cells, animals will be anaesthetised during the administration and will receive cells subcutaneously into one flank, normally the left (occasionally both for dual flank models), animals are expected to show only a mild transient pain from the implantation of the tumour and will recover quicky with no side effects.

Tumours will be allowed to grow and the mice will receive administrations of compounds at set time points, not exceeding the limits and frequency in the published guidance. Where animals are developing tumours as part of the study there will be limits on tumour volume and condition. Animals will be closely and regularly monitored and any animals that show one or more signs of ill health will be given supportive treatment such as warming, supplying extra bedding or enrichment. If there are no signs of improvement and before the severity limits are exceeded, the animals will be humanely killed.

The substances tested in this project and the procedures performed for the administration of the substances are expected to only cause mild, transient pain and no lasting harm, this will be similar to having a vaccination injection.

However, with some of the compounds, we may see unexpected welfare concerns when administering substances which have not been tested in vivo previously, for these studies we would run a small pilot with additional welfare checks to inform on appropriate care and to minimise the pain, suffering or lasting harm felt by the animals.

If adverse effects are observed in the study, a dose break will be implemented or the study stopped.

The least invasive route will be always used for administration and anaesthesia maybe used when appropriate to reduce any pain or suffering that the animal may experience. However, on occasions animals may exhibit signs of ill health following administration of substances, such as piloerection (bristling of fur), inactivity, hunching or unexpected weight loss.

Animals are not expected to show prolonged suffering and will quickly make a full recovery but will be given analgesics and post operative care to help with their recovery as required. Weight loss will be measured against the highest weight recorded for each animal and, in most cases, mice will be given dietary supplements to help the weight loss recovery before limits are reached.

Animals in our studies are not expected to die from our substances or our procedures, however in the event of an unexpected death, the carcass will be sent for postmortem to establish the reason for death and the incident reported so that a reoccurrence can be avoided in the future.

We will follow the published literature below from the vivarium, for example maximum volumes and administration of substances from A good practice guide to the administration of substances and removal of blood, including routes and volumes, K Diehl 2001 and published literature for clinical signs (FELASA Joint working groups) and any new guidance during the tenure of the licence to ensure the best welfare for our 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)?

The expected severity for most of the animals in our studies are mild based on our previous experience working with tumour growth models. Working to the published guidance, the majority of animals will reach end point based on tumour burden before showing moderate signs.

Mice: 70% Mild, 30% 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?

Tumour biology is profoundly influenced by its environment within the body, and the interaction between a tumour and the immune system cannot at present be reproduced in vitro due to the complexity of the interaction between the two systems that is currently not replicable by in vitro models.

Experiments assessing the effect of the body's metabolism on the drug (pharmacokinetics) also cannot be performed in non-animal systems.

In vitro assays are used to generate a proof of concept before the use of the drug in vivo. To improve translation into the clinic, human ex vivo tissue assays are used at an early stage in drug development to reduce animal research.

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

We have considered the use of in silico modelling, cell line work, in vitro cultures, organoid systems, and non-vertebrate animals.

We will continually monitor progress with non-animal alternative model systems by reading literature and attendance at conferences.

Why were they not suitable?

For in silico modelling, there are no datasets currently available that cover the experimental designs needed, but a key output of this project will be the generation of these datasets and in silico models.

For cell line and in vitro culture work, their systems do not adequately recapitulate the complexities of immune regulation. Certain validation experiments can and will be performed in cell lines and in vitro, however the in vivo experiments described here cannot be performed with the same degree of scientific accuracy without an in vivo system.

Organoid systems are showing increasingly interesting results at modelling single anatomical sites, however the study of immune responses in organoids is limited by the need for primary major histocompatibility complex (MHC)-matched donor cells and the lack of normal vascularisation.

Furthermore, these systems cannot recapitulate the pharmacokinetics and pharmacodynamics observed in a whole organism.

Added to this is the multi-organ nature of the questions here being assessed. Non-vertebrate animals do not have adaptive immune responses and therefore cannot be used in 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?

Our estimated animal numbers for the application are 17,500 mice and is based on previous numbers required to perform in vivo studies by the relevant teams on other licences to allow 1-2 drug programs to run per year and within the 5-year duration of this licence.

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 in our research, in the first instance we always investigate using in vitro or in silico methods (alongside extensive literature searches) and use this information to justify a need to progress the investigation into a mouse model. The minimum number of animals required to achieve a meaningful study to inform a significant biological effect will be determined based on power calculations.

Study designs will also include positive and negative controls, where relevant. We will continue to look at all the available data, both in literature and from our own data sets and records, to inform on numbers to ensure the minimum numbers are used whilst still producing robust data. Where new models are used, the use of pilot growth studies will provide statistical data to inform on numbers needed as part of the model development.

In this project, we are looking to use to the Fine Needle Aspiration (FNA) technique. This technique has been widely used with no adverse effect to the animal's welfare. Utilizing this technique, we will be looking to use less animals in our studies as we will not need to harvest samples at a terminal timepoint to understand the effects of our treatments on the tumour microenvironment.

Micro-sampling, introduced on our previous licence, reduced animal numbers needed on pharmacokinetic studies by 75%. By taking smaller volumes up to 20ul at an individual timepoint this means we gain up to 7 timepoints within the same animal across the whole study. Historically this would have to be done across 4 animals, as the volumes collected were larger to obtain the same data. By using micro-sampling technique, this increases the robustness of the data output due to minimum variability across mice.

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

Where historical data is not available pilot or growth/model development studies will be used to gather data, for example tumour growth rate data to optimise and minimise the number of animals needed for the study.

We will use the Fine Needle Aspiration (FNA) procedure with our mice enabling us to reduce the number of animals on a study as we will be able to take samples from two time points in life, which will reduce animal number.

Tissues will be taken from animals at end point, this is to ensure that we get the most from each animal and to reduce overall 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.

Mice aged 10 to 14 weeks with developed immune system will be used as their immunological responses and tumour development bears a high degree of similarity to that of humans. The genetic background and immunoreactivity of mice is very well characterised and there are a wealth of reagents and research tools available that are compatible with mice.

Murine tumour growth models yield reproducible results from our previous studies as well as from similar immunological tumour studies from other labs, making the mouse an ideal model for tumour growth studies to develop immune-oncology drugs where we are only able to test our therapies using in vivo methods.

The degree of research into mouse model welfare suggests the procedures within this project (e.g. tumour implantation, injection of drugs etc) can (and will) be performed with minimal pain and suffering caused to the animal so as not to exceed the severities of the licence. We will always use the least invasive methods and identify the earliest endpoints to reduce any unnecessary pain suffering or distress for the animals.

Any suffering due to tumour growth will be minimised with close monitoring of the animals. Provision of diet-gel food on cage floors will be added should animals have difficulty accessing diet and water due to mobility impairment. Analgesia will be provided where required. In a response to temperature drops, additional bedding or heat pads will be used.

Animals will be housed in social groups with access to food and water as well as additional cage environmental enrichment to prevent boredom, examples being fun tubes, chew blocks and various types of seeds to encourage foraging. Animal health and welfare will be consistently monitored and severities recorded in line with current welfare publications.

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

Adult mice are mammals with similar biology to humans which provides a more translatable model for tumour studies. Alternative models, for example zebra fish, flies and Nematodes, show a lower capacity to experience pain, suffering, distress or lasting harm and therefore their main advantage is in the refinement of the screening of new compounds before using more sentient in vivo models.

However, our use of in vitro assays already provides screening tools that avoid all animals.

Less sentient models, without an adaptive immune system, are unsuitable for immune-modulating or targeting drug development, and also lack literature for review in the field of immune-oncology, which would provide major challenges.

The current accepted research for a drug in preclinical development aiming for clinical trial application is strongly weighted to match the previous data in the field. Although some methods are in development to see if they can reproduce the accuracy of animal models,

in silico. Therefore, we plan to use both live mice and their tissues to gain evidence for developing our drugs towards clinical trial application whilst producing datasets for alternative in silico methods.

Terminally anaesthetised animals would also not be suitable candidates as the animals need to be alive to follow a time course to show the actions of the therapy.

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

Additional advice from named persons will be followed, especially for new techniques or technical refinements, when planning experiments. I will also consult relevant published guidelines, including the PREPARE guidelines. I will also be advised by the NC3Rs online resource library for the most up to date information on refined procedures, including dosing or sampling techniques.

Personal licence holders will attend training and keep their training up to date and follow published guidance for example the NC3Rs and will implement any refinements to ensure the welfare of the animals is optimal.

As implemented in the previous licence, cross-functional input will be gained from our scientists in a number of fields of expertise, including pharmacology, biomarkers, bioanalytical, PK/PD modelling, thus, ensuring that study plans are peer reviewed and aligned with project needs.

The micro sampling a technique mentioned in the reduction section showed that where repeatedly small amounts of blood are sampled from an individual animal (up to 20ul blood sampling) it reduced physiological stress by reducing the amount of blood taken (20ul vs ~70-100ul normal sampling technique).

Our clinical observations are performed no less than 2 times per week for each animal. This involves a visual check, body weight and tumour measurement, and these observations are performed by fully trained staff with digital callipers and without anaesthetic by scruffing the animal. However, there may be occasions (e.g. new models) where the animals may need to be anaesthetised, such as for early tumour growth measurements via palpitation where the tumour is at its smallest and it is a challenge to get an accurate measurement on a conscious mouse.

Once tumour-bearing animals have tumours reaching 10x10mm3, clinical observations will increase to 3 times per week until end point (15x15mm3 or equivalent with reduced limits on weekends based on predicted tumour growth) to ensure that the severity limits of the licence are not exceeded. Where relevant, tumours will be studied at the earliest stage of growth compatible with a meaningful result to the experiment.

All our animals will be provided with additional enrichment on top of the standard cage enrichment, along with additional bedding material. This will comprise of chew sticks and fun tubes as well as a rotation of seeds for foraging. Lone housing will be avoided unless it is necessary due to fighting, where the dominant animal will need to be separated. On these occasions, the animal left alone will be housed for the shortest period possible and given extra enrichment.

Any animals showing adverse effects or weight loss will have clinical observations performed daily until recovery. Animals not showing signs of recovery or likely to exceed the severity will be culled via an appropriate schedule one method. For unexpected death,

a post-mortem examination will be performed to investigate the reason for death. In either of these instances the NACWO and/or NVS will be informed.

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

Unless otherwise justified, studies will be carried out to best practice as described in Workman et al (2010). All experiments will integrate refinements from the NC3Rs (e.g., the PREPARE and ARRIVE guidelines), the LASA aseptic guidelines, LASA Diehl guidelines on volumes and frequency limits and advice from the local NIO or NVS.

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

The project licence holder and the In Vivo Team will continue to stay updated with our field of research through collaborations, conference attendance (IAT & LASA amongst others) and reading the literature (NC3Rs website and NIO newsletters). We will take particular note of any technical advances that enable reduction, refinement or replacement in our experimental design and attend regional 3Rs symposia.

The animal facility is a key source of knowledge, transmitting the latest information on the 3Rs to the team and researchers, and our internal protocols are shared across the institute, enabling rapid uptake of any improvements to the method across groups.

The project licence holder has a place on the Animal Welfare Ethical Review Board and will actively contribute to these meetings as well as having regular contact with the named persons in the animal unit NVS, NACWO and NTCO and regular updates from the home office/ASRU bulletins.

Advances in the 3Rs will be incorporated into the studies at the design stage, by informing the scientists designing the studies on this project, and at the implementation stage, by informing those carrying out the hands-on in vivo work. This will be communicated at regular team meetings within the in vivo team and to the animal unit staff.

17. Mechanisms of inflammation, resolution and repair in lung diseases and infections.

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

Respiratory, Infection, Lungs, Allergic inflammation, Non-allergic 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?

To assess how inflammatory cells affect lung health and function during disease and 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?

We do not fully understand how inflammatory cells arrive in the lungs and become activated. However, we do understand that there are certain important bottle-necks, for example a dependency on platelets (a cell type normally necessary for blood clotting), and their own accumulation in the lungs that affects other inflammatory cells. There is a strong medical need to better understand these processes because current anti-inflammatory drugs have sub-optimal activity and can have serious long-term side-effects for patients. Separately, there is a clinical need to understand how the immune system might be modulated in patients that have compromised immune systems during infection. The

processes we are discovering require animal experimentation because this is the only way of understanding the importance of various targets for future drug treatments that modulate inflammatory cell activation and that is controlled in a multi-organ, dynamic manner.

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

Our scientific discoveries that will be published in open-access peer internationally recognised reviewed journals, and communicated to target audiences. This data will be applicable to the field of respiratory diseases (e.g. asthma, chronic obstructive pulmonary disease COPD), infections (e.g. bacterial pneumonia), and pulmonary disease exacerbations. We will gain an understanding of the pharmacology of novel chemical compounds and also existing drugs for novel uses. This data will lead to the submission of patent applications and possible further pre-clinical development of novel drugs arising from the identification of new targets/pathways that regulate inflammation and infection. It is also possible that new transgenic animal models will be created to provide new tools to further elucidate disease mechanisms.

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

Academic: We are at the leading edge of research in platelet biology in the context of airway inflammation and further understanding of the way these cells underpin inflammatory cell recruitment will increase the scientific knowledge base more widely. Our observations and in vivo methodology have already been used by investigators in other fields to show the importance of platelets in with functions that are not related to blood clotting.

Industry: We have close links with the biotech and pharmaceutical industry, and governmental departments (e.g. MHRA, UKHSA, MOD). Our group is recognized internationally for research in the respiratory field and also investigating the role of platelets in inflammation and host defence. Indeed, we have successfully developed a new type of medicine for the treatment of respiratory disease which has resulted in the creation of a drug company (http:www.veronapharma.co.uk) and the drug has recently successfully undergone phase III clinical trials in patients with COPD. We are also identifying other novel potential targets on platelets to suppress inflammation, as well as investigating repurposing of drugs and drug combination to overcome microbial resistance and be effective in the treatment of stubborn lung infections, as well as in the treatment of pulmonary diseases during periods of worsening. The potential for developing a novel therapeutic intervention to treat respiratory diseases would be of significant interest to UK industry. This work therefore has the potential for substantial economic impact.

General public: Understanding the underlying mechanisms that give rise to the pathophysiology of asthma, chronic obstructive pulmonary disease (COPD), lung fibrosis, and respiratory infections would have a significant public health impact. We have a track record for developing novel medicines for the treatment of asthma and COPD and in communicating our research to the wider public who are keen to see the development of alternative anti-inflammatory drugs to corticosteroids.

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

We will seek to maximise the output of this work by continuing to collaborate with both academic colleagues and industrial partners in the respiratory field to share methodological developments and encourage researchers and academics to visit and train within our laboratories.

Data will be published in open-access peer reviewed scientific journals alongside presenting out work at both national and international symposia such as the European Respiratory Society and the American Thoracic society that recognise the need for research involving animals to be compliant under the ARRIVE guidelines.

New methodologies or animal model refinements and associated unsuccessful approaches will be published in specific journals dedicated to providing detail for techniques to be copied elsewhere.

When applicable, tissue can be stored long term (e.g. paraffin embedded sections, tissue stored at

-80c) for others to use in the future.

Species and numbers of animals expected to be used

- Mice: 13,500

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 physiological importance of the complex mechanisms that underly inflammatory cell activation and recruitment can only be ascertained in the whole body where blood function and , vascular, immune, and neurological systems are fully recapitulated. As it is these multi-tissue/organ/cellular systems determine how platelets and leukocytes become recruited to inflamed tissue from their remote origin.

Furthermore, measurement of parameters of lung function applicable to changes occurring in human airways can only replicated in whole body systems. Mice represent the lowest mammalian species that replicate the appropriate physiology when compared to human disease, with the existence of well characterised mouse models, of respiratory inflammation and infection, whilst the use of transgenic technology in mice allows the study of disease mechanisms with greater clarity. We will therefore use Inbred wildtype or genetically altered mice for this project. We will also use adult mice in these studies as they have an already developed immune system and are of a suitable size to allow surgery associated with our research objectives alongside the measurement of lung function changes.

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

Animals will be administered a therapeutic agent or cell depleting substance, or a sham control substance, either systemically or locally to the lungs. Animals will then be exposed to an allergen, sterile inflammatory insult, or inoculated with bacterial pathogens, usually directly to the lungs under recovery anaesthesia. This usually occurs on one occasion for an experiment that lasts up to 96 hours but can be repeated to induce a robust response or chronic inflammation (2-8 weeks of exposure).

Animals typically experience 4-5 procedures within a protocol (including but not limited to induction of inflammation/infection, administration of substances, blood sampling, measurement of a physiological process under terminal anaesthesia, termination). Less

than 5% of animals will experience a further 2- 3 procedures, and rarely, this may be prolonged if whole body irradiation followed by reconstitution of bone marrow is necessary. In this case, the duration of the experiment would be no more that 20 weeks, to take into account any necessary recovery periods.

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

Other than the disease phenotype, no general abnormal effects are expected. Mice injected with substances, bacterial pathogens, inflammatory insults, or blood sampling are subjected to restraint and needle injections, but these stresses are transient. When depleting animals of platelets or administering anti-platelet drugs , the mice may bleed for a longer duration of time, but this is finite (typically bleeding for 8 minutes compared to 3 minutes on average) and only occurs after trauma (for example peripheral blood sampling). Importantly, from our own research experience we do not observe adverse events that would be associated with substantial blood loss, because the blood loss is slight with the experiences the animals have. Some disease inducing agents will lead to weight loss approaching 15-20% over the timescale of the experiment. These types of experiments where we observe weight loss are no longer than 72-96 hours in length and comprise <5% of all our procedures. In some experiments, mice undergo surgery (for example ovariectomy, or the implantation of a telemetry device or drug minipump). These comprise <1% of all our procedures, and animals fully recover after the surgery (48-96 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)?

Overall project: 70% mild, 30% moderate. Derived from:

Breeding and maintenance of GA mice: 90% mild, 10% moderate (Protocols 1 & 2). Protocols involving inflammatory or infectious insults:

70% mild, 30% moderate (Protocol 3).

70% mild, 30% moderate (Protocol 4).

70% mild, 30% moderate (Protocol 5).

Tissue and blood harvesting protocols: 100% mild/non-recovery (Protocol 6).

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 research will be undertaken using murine models systems of airways inflammation. There is no alternative to these animal experiments since the scientific questions of this study have to be addressed in vivo to understand cell to cell interaction and physiological responses. This requires complex coordination between multiple organs and tissues for the migration of cells to inflammatory sites and the pathological consequences that has not been recapitulated in vitro and is currently too complex to model using in silico approaches. To study the consequence of lung inflammation on lung function requires the use of pulmonary function tests which cannot be studied in vitro. Cell activation, migration and function can be studied in vitro using cell culture techniques or peripheral inflammatory cells isolated from human donors which we use in our research to better understand isolated mechanisms or activation pathways. However, the complex mechanisms underlying inflammatory cell migration into inflamed tissues, and the overall importance of these can only be ascertained using in vivo model systems.

In order to investigate the mechanisms contributing toward the increase in airway irritability observed clinically in respiratory disease during pulmonary exacerbations also necessitates the use of animals. There is currently no replacement alternative to investigating these objectives in this licence. The phenomenon of airway irritability can only be observed in an intact mammal. Previous studies have shown that this phenomenon cannot be replicated in vitro even in human isolated airway preparations from individuals who have died of asthma or COPD.

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

Use of primary cell lines to recapitulate the lung vasculature, combined with technologies to provide circulating flow akin to blood. Use of primary peripheral inflammatory cells from both healthy human volunteers and patients with asthma and COPD to investigate how these cells become activated and function.

Why were they not suitable?

These techniques are used in our laboratory to inform us of isolated mechanisms pertinent to cell function. However these techniques are not suitable to measure: 1. Downstream consequences at the organ level (e.g. lung function, lung damage and repair, immune system modification); 2. The overall physiological importance of the mechanism of interest; 3. The contexts in which effects are observed due to interactions at the multi-organ level that require integration (e.g. immune system, central nervous system, mobilization from and recruitment to distinct areas of the body) as a dynamic system that responds differently as the disease phenotype progresses. 4. Long term effects of lung inflammation, lung infection and/or of substance administration (chronic 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?

Currently, we have 1 strain of transgenic mouse, and this may increase to 2-3. The transgenic strain is expected to be used throughout the 5-year period with up to 500 per

year produced. The numbers therefore also allow for the introduction of a limited number of new strains that have been identified.

Numbers of mice on experimental protocols is determined by the number of projects we expect require in vivo experimentation per year, the number of studies per year, and the cohort size of each individual study design, which are typically 20-50, depending on the number of groups and length of the study.

Cohort experiments may have one intervention/comparison measured (e.g. drug effect- this is 'one factorial') or several interventions/comparisons measured (usually two e.g. drug effect with platelet- depletion, termed 'multi-factorial') if using transgenic colonies, and include 1. sham control group(s); 2.disease inception group(s); 3. experimental intervention group(s); 4. positive control group. Control groups are necessary to understand basal processes, cell numbers; and positive control groups are sometimes included to ensure the responses are expected. For less well-established study types, a preliminary pilot study may be conducted where small numbers of animals may be used to generate data in order to ensure that the experiment operates to expectations and to generate data which may be used to optimise the study design for future experiments.

We anticipate using the equivalent number of sham or vehicle treated animals to the number of animals undergoing active treatment as we do not wish to rely on historical controls or an unbalanced experiment.

Group sizes are determined from the magnitude of effect expected, and variability from our previously published work or others.

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

Experimental design is discussed and agreed between researchers, and with the Principal Investigator (PI) who has also taught experimental design to MSc level (2011-2022), using guidance and publications from the NC3Rs website. The PI and the wider research group have successfully trained 40 PhD students, all of whom have worked with experimental animals and had their research recognised in high quality peer reviewed journals. The senior researchers each have numerous years experience in experimental design, and have the perspective of review from peer-reviewed internationally acclaimed journals. In particular, as Members and Fellows of the British Pharmacological Society, we are cognizant of best practice required for publication with regard to experimental design, analysis, and reporting:
https://bpspubs.onlinelibrary.wiley.com/hub/journal/14765381/declaration

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

The Mouse Colony Management System (MCMS) developed by the Sanger Institute is used. This allows efficient control and interaction between users and welfare officers/animal technicians of colonies. Efficient breeding procedures are used with advice from experienced Biological Services technicians and teaching resources from NC3Rs

Where possible, researchers do share experiments to maximize obtainable tissues for different research purposes from any individual animal (for example use of blood, tissue).

Studies are blinded, and animal allocation to groups randomized to ensure experimental bias is reduced in recordings or shared between groups.

Pilot studies are undertaken to observe dose-effect ranges of agents not previously 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.

Mice have been chosen as the species of choice as they have an immune system of comparable complexity to humans and due to the wide availability of reagents and genetically modified strains to investigate mechanism of cell activation and recruitment. For these reasons, mice are the most frequently used animals in studies of inflammation in the respiratory tract. Despite their small size, techniques have been developed in our laboratory that permits the recording of pulmonary lung mechanics, and cell rolling/adhesion in vascular beds using an intravital microscopic technique. Their small size also permits the visualization of inflammation using a variety of imaging modalities that would otherwise be challenging in larger species. The availability of reagents to label specific murine proteins allows one to more accurately follow the fate of an inflammatory response.

The models of lung inflammation and infection we use ensure mice are dosed with levels of inflammatory mediators, allergen or inoculated with bacteria titres that are limited in that the inflammatory response either diminishes over time (self-resolving) or does not progress with severity when used for short (acute) studies to investigate inflammatory cell recruitment. The models often require single daily intranasal or intracheal administrations of disease inducing agent and are very adaptable in that we can minimize the number of occasions that mice receive the inflammatory or bacteria insults to suit the aims of each individual experiment. The protocols are also well established, and the data available in the wider literature also helps to optimise our experimental design when determining the overall length of the experiment and number of exposures. We currently administer the inflammatory or infectious insults, under recovery anaesthesia for restraint purposes. This has meant we have moved away from inhalation exposure that required using boxes to house the mice for considerable periods of time (around 30 minutes per occasion), in a moist environment due to the nebulization of the compound (e.g. allergen).

Some experiments may require the recording of physiological parameters; and through the use of non- invasive recording equipment (e.g. remote telemetry) these recordings can be made over time with minimised interference of the animal (after initial surgery).

Other experiments may require the irradiation of mice to remove the bone marrow. However, the dose of radiation is limited to achieve the physiological effect required.

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

The structure of the respiratory system in non-vertebrate animals differs substantially from vertebrates, which makes them unsuitable for measuring lung function or cellular recruitment processes that are affected by anatomy. Rodents are among the lowest vertebrate species with lungs similar to humans that regulate breathing, gas exchange,

airway smooth muscle contraction and relaxation, blood flow (pulmonary and bronchial), inflammatory cell recruitment, mucus secretion, mucocilliary clearance and, airway surface liquid (tension) which is conserved across mammalian species and these parameters are all affected during disease. Mature (adult) animals are used because they will have a developed immune system and lung structure, that are necessary to understand therapeutic effects and physiological recording respectively.

Animals are terminally anesthetized for lung function measurement, intra-vital microscopy, cell tracking, measurement of bleeding, stimulation of nerves, and lung lavage sampling.

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

Where possible, we will seek to employ non-aversive handling, provide habituation to handling and provide appropriate environmental enrichment to reduce stressed, improve animal welfare and thereby decrease experimental variability increasing reliability and reproducibility. Dosing and duration of inflammatory of infectious insults to reach desired endpoints is conducted through pilot studies and this helps ensure severity of the procedure is limited to the minimum required. We will continuously monitor animal welfare following recovery procedures and adjust protocols to ensure the minimum exposure to harmful stimuli is used to achieve a significant result using scoresheets (eg. Body Conditioning Scoring) when a known affect is likely to occur, or a novel combination of procedures are undertaken to reduce delay in humane of husbandry decisions. Post operative care and the use of analgesia, improvements are made through discussion with the NVS.

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

We will compare our protocols to other published disease models to evaluate how we can incorporate refinements when they are developed. We continue to use the NC3Rs resource to check for developments. This is the most accessible resource that promotes refinement in an explicit manner.

We will follow the published ARRIVE guidelines alongside prior established protocols and codes of practice including but not limited to the code of practice for housing and care of animals bred, supplied or used for scientific purposes, LASA guidelines, and Universities Federation for Animal Welfare (UFAW) guidelines and publications. Learned societies also publish monographs that allow good comparison and searches for refinement of appropriate disease models (for example the American Thoracic Society publish models of acute lung injury, COPD, respiratory exacerbations). Finally, we will continue to discuss and adhere to local AWERB standards to ensure all recent refinements are adopted to establish a clear scientific response allowing us to minimise the number of animals used whilst also improving the welfare of animals used in this application.

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

We will continue to review the literature for any new developments in experimental protocols, whilst staying up to date with other research guidelines in good laboratory practice and animal welfare through attending any relevant seminars advertised by the NC3Rs and access material available via the NC3Rs resource library as a group. An example of how this has been used is the refinement of our blood sampling methods with videos/documentation on the website. I am a subscriber to the NC3Rs newsletter and I am

therefore also updated with developments. We share and discuss options for refinement both within and outside of our group, including but not limited to staff within the institute's biological services unit and the NVS.

18. Mechanisms of synapse function 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

Synapses, Synaptome mapping, Synaptopathy

Animal types	Life stages
Mice	adult, juvenile, neonate, embryo, 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 the mechanisms that control the composition of synapses (specialised connections between nerve cells) in health and disease by

(i) monitoring synapses through imaging of brain tissue or living mice, and through isolating synaptic proteins and

(ii) by studying synapses in animal models with genetic mutations relevant to human disease and

(iii) by modulating synapses using drugs, diet or altered experiences.

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?

Synapses are the hallmark of the brain, found in vast numbers and connect neurons together into the circuits and networks that underlie all behaviours. Synapses are also

immensely complex at the molecular level. They contain thousands of proteins and hundreds of the genes encoding these proteins are mutated in over 130 brain disorders. Synapse proteins are also the targets of most therapeutic drugs used to treat neurological and psychiatric disorders.

A major advance in recent years has been the recognition that synapses are highly diverse at the molecular level. The numerous synaptic proteins are differentially distributed into individual synapses and as a result different synapses are affected in particular diseases. We developed a groundbreaking technology pipeline that enabled us to survey billions of individual synapses and assess their protein composition across the entire mouse brain at any age. This approach, known as synaptome mapping, provides a roadmap for understanding how genetic disorders give rise to their behavioural phenotypes. The protein encoded by a particular gene is found in a particular synapse in a brain regions.

The goal of this project is to leverage synaptome mapping, in combination with complementary genetic, proteomic and cellular approaches, to comprehensively analyse synapse diversity and architecture across the major classes of mammalian brain synapses. This provides the missing link between existing and emerging synapse classifications that is crucial to full functional understanding. Within this comprehensive, integrated framework, our programme will encompass the impacts of ageing, environment, lived experience and mutations, uncovering the contribution of synapse diversity to regional and temporal vulnerabilities, resiliencies and adaptation.

These findings will inform new principles of brain architecture and function, with important implications across all areas from genetics to systems neuroscience, and will direct novel avenues for therapies aimed at treating a wide range of brain and behavioural disorders.

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

1. The generation of new fundamental knowledge about the anatomy and function of the mammalian brain of direct relevance to human health and disease.

2. The generation of new principles about the mechanisms by which diseases and disorders produce their pathological changes in behaviour and physiology. Central to this is the identification of the “vulnerable synapses” that are targeted in each of these disorders.

3. An understanding of how and why pharmaceutical drugs target specific synapses in the brain, which is essential for understanding how they exert their therapeutic effects and side-effects.

4. An understanding of how brain imaging methods used to diagnose and monitor patients can be used to detect brain diseases involving synapse damage.

5. All our work will be published in peer reviewed scientific journals, presented at local, national and international meetings.

6. Our work also produces databases and brain atlas resources, which are made freely available on the web and integrated with other brain atlas resources.

7. Genetically modified mice of use to the scientific community will be deposited in repositories for open access.

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

Benefits in the short term:

1. Data will be freely and openly released before publication of peer reviewed academic papers during the course of the project via preprint servers, websites of brain atlases and databases. This information has wide application across many scientific and medical domains.

2. Genetically modified mice will be shared with collaborators directly and through repositories.

3. Results will be shared with collaborators enabling them to enhance knowledge through new investigations. For example, experimental data will be shared with computational scientists who use the data to model how the brain works.

4. Knowledge from our study will immediately inform on the interpretation of clinical studies of brain imaging.

Benefits in the longer term:

5. The identification of those synapses that are vulnerable in disease will drive new approaches to neuropathological examination of human and animal brain tissue and identification of synaptic targets of drugs.

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

Primary dissemination to the research community will be via timely preprint and journal publication and invited talks, plus a website providing synaptome data linked to genetic, genomic, transcriptome, electrophysiological, behavioural, connectomic and brain imaging databases, as well as technical information (e.g. community standards).

Synaptome biology is a new area of neuroscience and utilises novel laboratory and computational tools. In addition to open availability online, we will maximise resource dissemination by offering short training courses to the research community as a two-way skill-transfer process that will promote ongoing tool development and foster collaborations.

Species and numbers of animals expected to be used

- Mice: Mice: 15,000

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 mouse is the most appropriate, and established, vertebrate model of direct relevance to humans for studying synaptic architecture and function across brain regions and lifespan. We have used our synaptome mapping methods to examine all ages from birth to old age in mice and found differences at all ages providing fundamental insights into development, maturity and aging Murine genetic models facilitate understanding of the function of presynaptic and postsynaptic proteins in normal behaviour, learning and memory, and in neurological conditions such as Alzheimer’s disease and schizophrenia. We have created, and plan to expand, a unique panel of mouse knock-in lines encoding

fluorescent and affinity tags targeted to specific synaptic proteins. These generate extremely robust labelling for visualising the distribution and turnover of key synaptic proteins within and between synapses, using state-of-the-art microscopy.

Application of reduction and refinement 3Rs principles will ensure appropriate and responsible use of animal subjects; opportunities for replacement will be under constant consideration. Novel tools for genetic manipulation (CRISPR) represent a refinement measure that enables further information to be obtained from any one animal and a reduction of animals required for breeding to acquire intended genotypes. In vitro neuronal systems cannot inform on synapse type distribution between whole-brain regions or their relationship with the connectome. They are not readily amenable to combinations of genetic mutations and genetic tags, as readily achieved by intercrossing mice. Brain organoids currently recapitulate only early stages of brain development. In vitro systems are not suitable for studies investigating environmental impacts, nor for lifespan studies that need to recapitulate natural ageing and windows of disease susceptibility.

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

The severity of procedures is mild, moderate or non-recovery. Routine technical procedures will be subthreshold or non-recovery as they involve breeding and maintaining animals and culling by non- recovery terminal anaesthesia in order to harvest brain tissue.

Tissue sections for synaptome mapping are prepared from mouse brains acquired by perfusion-fixation under terminal anaesthesia, vital for preparation of sections of brain tissue that are cleared of erythrocytes and completely infiltrated with fixative to maintain fluorescent tags.

Moderate procedures specific to the project comprise the following. Behavioural manipulation including environmental enrichment (providing a more stimulating environment such as mazes, wheels and different cage levels), whisker stimulation, and learning paradigms such as cue and contextual conditioning (animals are placed in a box) and taste aversion (animals are trained to avoid a particular taste in water). Intraperitoneal injections of ligands for protein turnover analysis; surgery to allow direct injection of viral vectors into the brain to alter excitatory/inhibitory balance; dosing to label the ligand-binding tags on proteins of interest will cause only transient pain. The severity of these procedures cannot be lowered.

All surgery will be performed using aseptic techniques and analgesics will be given. Routes of administration, volumes and frequencies of dosing are not expected to cause any adverse effects other than the transient discomfort of injection as they will follow best practice guidelines. Animals will be monitored carefully. In all circumstances, any animal that shows clinical signs exceeding moderate severity will be killed by a Schedule 1 method or, in the case of individual animals of particular scientific interest, advice will be sought from the local Home Office Inspector.

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

The majority of animals under this project licence will be subjected to minimal procedures, for example may receive an injected labelling or pharmacological substance that we know from experience results in minimal discomfort to the animals. Some procedures will require surgery and anaesthesia, however pain relief will always be administered to reduce any unnecessary suffering associated with this. A small percentage of animals display post-

surgery complications, however, should this be evident animals will be humanely killed immediately.

The behavioural tests used are primarily not associated with any adverse effects, but some may require a small amount of food restriction to motivate search for food rewards, short term single housing which is known to be stressful, injection of substances that are moderately nauseating (but cause no long term adverse effects) or small foot shocks that are designed to cause only minimal, transient discomfort (aversive stimulus). All animals throughout these experiments will be monitored carefully and if there are any signs that the animals are under distress or unwell they will be humanely killed immediately.

As for all procedures, we expect no more than transient discomfort to the animal but careful monitor regimes will ensure that any animal that goes above that will be humanely killed. At the end of all experiments, animals will be humanely killed and brain tissue will be removed and used for imaging or extraction of synapse proteins. We expect minimal adverse effects as all of our protocols have a moderate expected severity and the majority of animals will only experience procedures of mild severity. Experiments that are of moderate severity, e.g. live imaging that requires anaesthesia, are important to translate our findings in mice to clinically relevant imaging approaches in humans. Throughout these and similar experiments animals will be regularly monitored to minimise any unexpected adverse effects and unnecessary suffering. The genetically altered mice which are developed to model human diseases primarily affect neurological behaviour and are not associated with adverse effects that impact negatively on animal welfare. In rare cases where adverse effects develop, animals will be studied prior to this time point. The animals will be humanely culled at the end of the experiments and the brain tissue will be used for imaging or extraction of synapse proteins.

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 procedures is mild, moderate or non-recovery.

We have based the estimate of the proportion of animals in each category based on the past 5 years of data obtained from the same experimental protocols included in this application.

Subthreshold: 50%
Mild: 15%
Moderate: 35%

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?

This project focusses on the synaptome architecture of the mammalian brain, which comprises the 3D distribution of billions of molecularly diverse synapses into an exquisite architecture in which every neuron and brain regions has a particular composition of synapses. The synaptome architecture changes continuously throughout the lifespan. The synaptome architecture cannot be studied in unicellular eukaryotes or prokaryotes because they do not contain synapses. Furthermore, invertebrate organisms are not presently suitable because the techniques for measuring synaptome architecture do not exist for invertebrates, the regional anatomy of the invertebrate brain is not homologous to the mammalian brain, and we are studying synaptic proteins which are unique to mammals.

It is currently impossible to understand how synapse proteins are regulated and re-distribute throughout life and in response to disease, or from altered experiences in anything other than a living organism. In our work we are actively pursuing alternatives to animal studies through the use of computational models or in vitro cellular model, which at the present time are too limited to substitute for whole animal studies, especially for behavioural studies.

Mice provide a valuable animal model because there is high conservation between the genes of humans and mice. That means that we can study human disease-relevant gene changes in mice to monitor the effect on synapses.

From studies in the mouse, we have learned that there is a 3D synaptome architecture of the brain arising from extensive synapse diversity. At the present time we do not have any cellular model system that would replace mice because these cellular systems (e.g. cultured neurons, IPS cell-derived organoids) do not recapitulate the 3D synaptome architecture.

We have learned that generation of mice carrying multiple genetic tags permits reduction in the number of animals used.

We have refined our behavioural testing by removing unnecessary tasks and have implemented an oral drug delivery protocol that removes the routine use of oral gavage.

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

1. in vitro neuronal systems such as induced pluripotent stem cells derived neurons or organioids.

2. Post mortem human brain tissue

3. Computational models of brain cells.

Why were they not suitable?

1. The appropriate 3D organization of interconnected neurons is essential for understanding the synaptome architecture. In vitro neuronal systems such as induced pluripotent stem cells derived neurons or organioids, although possibly of use in some of the translational steps (drug testing), only appear to faithfully recapitulate early stages of brain development, have lower cellular diversity than animals and the nerve cells are not spatially organized and connected as they are in the intact animal.

2. We are conducting studies on post-mortem human brain tissue in parallel to our studies of mice. Post-mortem studies of human brain are an important approach which

allows detection of pathology, but cannot substitute for animal experiments or live tissue preparations that are used to reveal molecular and cellular mechanisms of disease.

3. We are using computational models of brain function that have been directly informed by our experimental studies with the goal of ultimately replacing animal experiments. At present our knowledge of the biological systems are too limited and there are major limitations in the computational modelling of the highly complex synaptome anatomy. We expect to close the gap between the biological and computational approaches during the course 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 estimation provided above can be based on peer reviewed publications resulting from our many years of studies using the protocols in this application.

The generation of a new genetically modified mouse lines requires a lot of breeding, in particular where more than one genetic modification is required, e.g. for a conditional knock-out, or even just for re- derivation into a unit followed by breeding to homozygosity or where back-crossing to a particular genetic background is required. This results in the use of a large number of mice (>100 per line even with modern approaches)

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

We have applied NC3Rs principles to ensure appropriate and responsible use of animal subjects; sufficient animal numbers needed to achieve the result and opportunities for reduction will be under constant consideration. Our experimental design will be based on previously standardised assays and protocols where possible, or for novel approaches, based on pilot experiments guided by the literature or collaborators with expert knowledge. We will use the experimental design assistant EDA to support experimental planning with animal users. Novel tools for genetic manipulation (CRISPR) represent a refinement measure that enables further information to be obtained from any one animal and a reduction of animals required for breeding to acquire intended genotypes.

Previously generated data will be utilised to determine sample sizes where possible, and for all studies, power will be assessed post hoc and group sizes amended if the statistic assumptions are not met. Mice of both sexes will be utilised and all animal experiments will be carried out and reported in accordance with ARRIVE guidelines. In all experiments, the unit of analysis is the animal and we routinely employ blinding and randomisation of genotypes and/or treatment groups to avoid bias.

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

Mice of both sexes will be utilised and all animal experiments will be carried out and reported in accordance with ARRIVE guidelines. In all experiments, the unit of analysis is

the animal and we routinely employ blinding and randomisation of genotypes and/or treatment groups to avoid bias.

The experimental design will be based on previously standardised assays and protocols where possible, or for novel approaches, based on pilot experiments guided by the literature or collaborators with expert knowledge.

Previously generated data will be utilised to perform power calculations to determine sample sizes where possible, and for all studies, power will be assessed post hoc and group sizes amended if the statistic assumptions are not met.

Larger studies carried out under this project will be specifically developed in a step-wise approach where effect sizes and available complementary data sets (e.g. behaviour, transcriptomics) will be used to guide future studies for more focussed analysis. This approach will maximise the probability of detecting biologically relevant changes using the lowest possible number of animals. Experimental outputs for comparison between groups is alterations to synaptic proteins, either detected from protein extracts (biochemical approaches) or ex vivo/in vitro visualised proteins (microscopy) or in vivo live imaging. In-house bioinformatics staff employ standardised data analysis on the whole brain level to maximise the value of data collected per animal and reduce variation caused by bias and/or human error.

To minimise animal numbers we try to reduce the variation of our experimental data, this is done by

(i) using standard protocols and conditions assuring that outcomes can be compared between normal and disease-relevant mice as well as between different lines of disease-relevant mice,

(ii) using mice that are all of the same age and genetic background,

(iii) design studies that generate both structural (e.g. imaging) and functional (e.g. behaviour) data in the same group of mice

(iv) studying synapse proteins over the whole mouse brain rather than very small areas

The group has over 35 years of experience breeding GA animals and day-to-day maintenance will be managed by the laboratory and colony manager in consultation with the lead and the department to ensure optimal experimental design, breeding and maintenance strategies.

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 preferred animal model for this project for several reasons. Firstly, synapse proteins are highly conserved between mice and humans at the structural and functional levels. Second, mice are the best mammalian model of the human brain for molecular studies because more is known about the mouse brain than any other species and there are more tools for manipulating and studying molecular function. Third, the mouse brain is smaller and more amenable to synaptome mapping than rats and non-human primates. Fourth, there is a vast collection of genetically modified mice available in the scientific community that can be used (e.g. genetic models of human mutations) which do not exist in other mammalian species.

The method of synaptome mapping is a major refinement over other those methods to study synapses that require invasive and sometime chronic procedures, such as electrode and fibre-optic implants.

Synaptome mapping also allows brain-wide analysis, in contrast to all other methods for synapse examination that are only suitable for very small regions of the brain,

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

The mouse is the most appropriate, and established, vertebrate model of direct relevance to humans for studying synaptic architecture and function across brain regions and lifespan. Murine genetic models facilitate understanding of the function of presynaptic and postsynaptic proteins in normal behaviour, learning and memory, and in neurological conditions such as Alzheimer's disease and schizophrenia.

Alternative less sentient neuronal systems or In vitro models cannot inform on synapse type distribution between whole-brain regions or their relationship with the connectome. They are not readily amenable to combinations of genetic mutations and genetic tags, as readily achieved by intercrossing mice. Brain organoids currently recapitulate only early stages of brain development. In vitro systems are not suitable for studies investigating environmental impacts, nor for lifespan studies that need to recapitulate natural ageing and windows of disease susceptibility.

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

We will create genetically engineered mice by the insertion of protein tags that permit the visualization and purification of proteins for studies on synapse proteins and disease models that cannot be achieved by in-vitro methods or human tissue. The vast majority of tagged mutants do not display clinical signs of adverse effects: (eg locomotor impairment) and behave as wild-type mice of the background strain.

We do not expect that any additional lines created under the new PPL will display phenotypes with clinical signs of adverse effects. All new lines will be closely monitored for neurological and clinical (weight loss, skin, gait etc) and assigned to the appropriate Breeding Protocol. To date, our mice do not develop chronic clinical signs as they age but all mice held for aging studies will be routinely monitored for body condition.

GA mice that exhibit behavioural phenotypes modelling human brain disease(s) may be 'treated' with therapeutic compounds to investigate whether baseline behaviour can be restored or modulated. The welfare impact of pharmacological agents, routes of administration and doses will be tested in small- scale dose-finding pilot studies to establish safe & effective drug range and appropriate monitoring intervals which will be incorporated into larger-scale studies.

Neurotransmitter receptor agonists/antagonists such as kainic acid or ketamine may be used. Kainic acid induction protocols may result in epileptic periods – however when injected into the peritoneum at a single dose these are short-term and mice usually make a full recovery to normal behaviour. The doses and time-points of termination used are representative of current paradigms to produce sufficient levels of neuronal upregulation with neuroanatomical changes. Ketamine induction may result in hyperactivity at lower doses but a more sedative-like effect at higher doses. The effects are short-term and mice usually make a full recovery to normal behaviour. The doses and time-points of termination used are representative of current paradigms to produce sufficient levels of schizophrenic-like behavioural deficits with neuroanatomical changes. The welfare impact of other selected pharmacological agents, routes of administration and doses will be tested in small-scale dose-finding pilot studies to establish safe & effective drug range and appropriate monitoring intervals which will be incorporated into larger-scale studies.

Ageing mice will go onto our ageing protocol and be carefully monitored daily by facility staff for changes in weight, mobility, general health.

Surgery - All recovery and long-term non-recovery surgery will be done aseptically according to Home Office and local/published guidelines.

Analgesia - Peri-operative analgesia will be given and maintained after surgery for as long as is necessary to alleviate pain. Pain will be monitored by observation of abnormal behaviour/appearance;

e.g. hunched posture, isolation from group, piloerection, and analgesia supplemented if required. If pain persists, the animal will be humanely culled.

End-points – The majority of mice will be culled by a schedule 1 method or perfusion/fixation as detailed in the project plan.

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

To ensure our experiments are conducted in the most refined way we will use the following best practice guidance

PREPARE (Planning Research and Experimental Procedures on Animals:Recommendations for Excellence): guidelines for planning animal research and testing

As part of ongoing efforts to reduce waste, promote animal alternatives (all the three Rs-Replacement, Reduction, Refinement), and increase the reproducibility of research and testing, a group of experts from the UK and Norway, led by Norecopa,(Norway's National Consensus Platform for the advancement of "the 3 Rs" in connection with animal experiments) has produced a set of guidelines for planning experiments: https://norecopa.no/prepare

The NC3Rs, a UK based scientific organisation that works nationally and internationally with the research community to replace, refine and reduce the use f animals in research and testing: https://nc3rs.org.uk/

Laboratory Animal Science Association (LASA) The UK society for scientists and laboratory animal professionals with an interest in animal research, 3Rs, care and welfare,

education and training, ethics and policy and regulation of animal research: https://www.lasa.co.uk/

We will follow best practice guidance for frequency and administration of substances for example those outlined in LASA good practice guidelines. In terms of surgical asepsis we will similarly follow 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?

We will stay informed about advances in the 3Rs by regularly checking information on NC3Rs website. We've also signed up to the NC3Rs newsletter and plan to meet the NC3Rs Regional Programme Manager and attend their symposia. We will attend informational seminars and events held locally, and where applicable change our practice accordingly.

19. Neuroendocrine regulation of energy balance

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

obesity, metabolism, body weight, food intake, hypothalamus

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?

Investigation of novel hypothalamic pathways in the brain that alter energy balance and metabolic phenotype in mice.

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?

Overweight and obesity are leading contributors to preventable death worldwide, with comorbidities including, heart disease, stroke, type 2 diabetes and some cancers, as well as worse outcomes and higher mortality from Covid-19. The estimated global cost of overweight and obesity is similar to the combined global cost of armed violence, war and terrorism. The regulation of energy balance (the balance between energy stored as body fat; ingested energy/appetite; and energy burned in metabolism and activity) occurs largely through brain hormonal regulation, particularly in the hypothalamus. This has been established for over a century through early lesion experiments, and in recent years human genome-wide association studies have identified many genetic variants associated

with body weight that exclusively act through hypothalamic pathways. Despite this, there are currently no successful pharmacological interventions that target hypothalamic pathways, with those previously developed having intolerable side effects. Therefore, it is imperative that to tackle the obesity epidemic, novel pathways regulating energy balance must be investigated to target and develop new interventions.

My recent work has identified a novel transcription factor that regulates body weight in mice, accompanied with altered hypothalamic gene expression in specific subregions of the hypothalamus. The proposed work will build on this through 1) metabolic phenotyping of mice fed altered diets (e.g., high fat, high protein, or other specific dietary composition, with appropriate control diets) followed by investigation of brain region specific gene and protein expression to identify target pathways, and 2) through conditional manipulation of gene expression in selective brain regions, followed by metabolic phenotyping and post-mortem investigation of gene and protein expression.

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

This work will generate new knowledge on the understanding of how brain and hormone systems regulate body weight through appetite and metabolism regulating pathways, and will be disseminated through annual conference presentations at national and international conferences and published in international peer reviewed journals. This work will be communicated to the wider public through press releases and articles in online news forums such as The Conversation, which are then picked up by online news outlets, and public engagement activities locally and nationally.

Tissues collected from the animals used in these investigations that are not used in initial investigations (i.e. not the primary output from the study) will be collected and stored appropriately for future investigations in order to maximise outputs.

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

These outputs will benefit the establishment of the PPL holder’s research group and the members of this group, as well as the broader scientific community both locally, nationally, and internationally.

Communication of these outputs will allow advancement of the understanding of neuroendocrine regulation of energy balance worldwide, benefitting not only the research community and the advancement of their research but may lead to development of new treatments for people affected by obesity and metabolic disease such as type 2 diabetes and cardiovascular disease, as well as the many conditions that are adversely affected by being overweight ad obese.

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

All research outputs will be published open access and made available public repositories. Attempts to publish all data will be made, and the PPL applicant has successfully published “negative data” in the past as evidence of this. Large datasets arising from e.g. sequencing data will be made publicly available to other researchers to maximise the impact from these animals (reduction).

The work will be presented regularly at national and international conferences to disseminate to the wider scientific community. Public communication of the work will be carried out through local and national initiatives such as Café Scientifique, Pint of Science, and through online news articles for example in The Conversation and press releases will

be generated to accompany publication of original research articles. Tissues not used in initial investigations will be stored appropriately and shared with other investigators to make full use of all samples generated in these studies.

Species and numbers of animals expected to be used

- Mice: 360

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-defined model for the regulation of bodyweight, metabolism and growth. The mouse has well conserved physiology with humans and other mammals, including regulation of glucose homeostasis and hypothalamic neuronal pathways demonstrably involved in energy balance. Existing genetically altered mouse lines can be taken advantage of to manipulate gene expression in specific brain regions and/or cell types in specific brain regions to identify pathways driving physiological changes. There is currently no better alternative model for such investigations.

Mice must be used from juvenile to adult to investigate the whole-body regulation of growth, appetite and metabolism. Metabolic rate, food intake behaviour and the hormonal communication between multiple different organs of a living animal can only be investigated in the whole animal. This requires a mammalian, endothermic species to investigate interaction between different biological systems in the body. Since body weight regulation, obesity, growth effects and metabolic disease like type 2 diabetes are all progressive conditions, these experiments are required to be carried out over time (weeks to months) to assess cumulative effects of altered diet and gene expression. Genetic manipulation in specific brain areas are done in adult mice (by intercranial injection of a virus or substance) to avoid developmental effects of using a congenital knockout model. This allows for a more refined investigation.

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

Typically, mice will be randomised and given free access to an altered diet (e.g. 60% fat, or low fat nutrient matched control diet) and where possible will be pair-housed with another mouse of the same sex. Mice will be weighed weekly, and have food weighed to calculate food intake for duration of experiment.

Mice may receive a single or bilateral intercranial injection under anaesthesia of a virus or substance that alters gene expression in an identified area of the brain.

Mice will then undergo a metabolic phenotyping programme which typically involves:

- Body composition analysis, once in every two weeks (12-14 weeks)

- Non-invasive urine collection (twice)

- Food withdrawal up to maximum 16h (three times - once for fast/refeed, once for blood sampling, once for tolerance test)

- Blood sampling by tail bleed. (once)

- Glucose (oral, intravenous or intraperitoneal;) or insulin (intraperitoneal) tolerance test. Glucose administered. Followed by multiple small blood sampling by tail bleed. (once)

- Mice may be exposed to altered light cycles (circadian disruption) for up to 8 weeks. Typically this could be a shift in timing of lights-on by several hours, or housing in constant darkness, or exposure to dim light at night.

- Non-invasive assessment of metabolism (energy expenditure, respiratory exchange ratio) and activity using home-cage systems. (typically, single housed in promethion home cage for one week)

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

Mice that receive an intercranial injection are expected to make an unremarkable recovery following surgery and will receive pain relief and post operative care.

It is anticipated some animals may experience minor transient discomfort during this project, resulting from the administration of substances, food withdrawal, and blood sampling.

Mice are expected to gain weight with high fat diet, and other diets may slow down normal weight gain. Some genetic alterations may lead to slowed weight gain, or weight loss. This will be monitored with weekly weighing and daily cage monitoring as part of routine husbandry. High fat diet can cause a greasy coat and the mice may overgroom to compensate for this, so mice will be monitored for signs of ill effects.

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 small number of mice will have procedures performed under terminal anaesthesia and will not receive any other procedures (non - recovery). 10%

Approximately 20% mice will experience mild severity.

Most mice will receive a combination of procedures that would individually be classified as mild, but because of the combined procedures may be classified as moderate. Some mice will receive surgery (intercranial injections), and this is a moderate procedure. Combined with additional mild procedures, after a period of recovery, the maximum severity for these animals is also moderate. 70 %

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 systems and diseases I study are systemic and multi-organ systems that cannot be studied in alternative models, while use of in vitro and in silico tools will be used to maximise impact and understanding of this project, bodyweight and metabolic processes involved with brain and hormone interactions cannot be studied in anything less than a whole animal.

The physiology of the mouse is very similar to human, and the mechanisms and hormones that regulate this physiology is also very similar to in humans. The mouse is the best species to use to study these mechanisms and develop new interventions for human disease.

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

Molecular and cellular experiments are used in addition to animal experiments to underpin and extract findings that do not require an animal model. They are used to validate substances that will later be used in animal studies. Likewise, where in silico methods are available, these are used before considering animal experiments. This allows in vivo work to be properly informed and performed as safely and correctly as possible.

Why were they not suitable?

The use of an animal model allows for investigation of whole-body physiology and behaviour. This is not possible to do in a non-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?

Estimates are derived from previous studies I and my previous lab groups have carried out, and those published in the scientific literature.

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 Assistant is used in the design of all animal experiments and this is included in all grant applications that include animal experiments. This makes sure that all considerations are made to appropriate experimental design. Sample sizes for each experiment are calculated using power calculations, taking pilot data from previous experiments to calculate the minimum number of animals required for each experiment.

Experimental design is based on our extensive experience and the use of pilot experiments where appropriate. For food intake measurements where animals are pair housed and food is manually measured, the experimental unit is the cage, rather than the animal. This means that power analyses are carried out with this in mind and this reduces variability in the results. Male and female mice will be used where possible, and this will be taken into account when statistically analysing the data to reduce risk of variability.

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 randomised and blinded where possible to treatment and planned in accordance 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?

Both sexes will be used in these studies. Pilot studies will be performed where required to assess efficiency of surgery and genetic recombination, as well as optimal route of administration, before moving on to 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 - wildtype and genetically modified mice.

Substances are prepared and administered using sterile technique. The route of administration is via the least invasive method appropriate to the model. The volume of substances to be used will be in accordance with the Laboratory Animal Science Association (LASA) good practice guidelines.

Substances will be administered intracranially in a sterile manner under general anaesthesia. Animals will receive analgesia pre and post-surgery in order to mitigate pain and discomfort.

For the metabolic assessment of mice in vivo we employ the Sable Promethion system. While the mice live in their home cages as food and drink intake can be monitored without any disturbance to the animals. Mice are individually housed for this procedure and male mice will not be grouped housed again following, therefore where possible, this procedure will occur at the end of the experiment and mice will be humanely killed following this procedure.

Blood sampling will be performed using sterile techniques and volumes collected will be in accordance with the LASA / NC3Rs guidelines. We will aim to take the smallest volume which will allow for adequate analysis.

All animals will be humanely killed a Schedule 1 method

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

Mice are the least sentient species that will allow us to achieve our objectives. Mice are a well-defined model for the regulation of bodyweight, metabolism and growth. The mouse has well conserved physiology with humans and other mammals, including regulation of glucose homeostasis and hypothalamic neuronal pathways demonstrably involved in energy balance. Mice and humans share 97.5% of their DNA sequences, making mice preferential to using less sentient species such as zebra fish or drosophila.

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.

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 research and testing) are used to better support the preparation of animal studies. The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments), NC3Rs and LASA Good Practice Guidelines are also to be incorporated.

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

We will maintain close interactions with the relevant welfare, training and information officers as they oversee and perform in vivo studies. The PPL holder will stay informed of advances in the 3Rs by regularly checking the NC3Rs webpages (https://nc3rs.org.uk/the-3rs) and the newsletters which are circulated monthly. Moreover, the PPL holder will attend appropriate seminars, symposiums and conferences deemed suitable.

20. Regulation of the immune response with respiratory infections

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, Respiratory, Therapy, Lung disease

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

This project aims to characterize the essential features of the immune system that protect against respiratory infections, to identify why some chronic diseases make people more susceptible to infections, why infections can cause worsening of existing disease, and to develop and test therapies that improve defence against infection and/or reduce 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?

Respiratory infections are a leading cause of global mortality and disability, especially in at-risk groups. Infectious diseases are associated with more severe disease in at-risk groups including people with chronic lung diseases, such as chronic obstructive pulmonary disease (COPD), or the elderly.

Conversely, exposure to infections worsen chronic diseases. For example, exacerbations of COPD, which are frequently due to viral or bacterial infections are associated with acceleration of disease progression, hospitalization, and death. Conventional treatment of infections relies on antimicrobials, such as antibiotics for bacteria, but this is limited by the emergence of antimicrobial resistance and novel pathogens. An alternative approach is to target the host. To develop an effective host directed therapy we need a better understanding of why the host response is dysregulated. We need to be able to understand how this contributes to the harmful consequences and which ones are protective. We can then target these with therapeutics that minimize unwanted effects.

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

This project is expected to identify parts of the immune system that are important in regulating the response to pathogens and how these are impacted by pre-existing conditions that are associated with worse outcomes after infection. This knowledge will lead to the development of therapies to enhance pathogen killing and/or reduce inflammation. We will identify the most selective targets with which to downregulate the harmful aspects of the inflammatory cell response whilst not compromising host defense to other pathogens at different sites.

The outputs will form parts of peer-reviewed articles, presented at meetings and parts of PhD students thesis deposited in online repositories.

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

The outputs will be of short-term benefit to my group and other researchers. Targets and associated therapeutic compounds will be further investigated in grant applications and future projects, with the aim to translate the studies into clinical trials and human studies and thus will be of medium-term benefit to industry and long-term benefit to patients by reducing morbidity and mortality from respiratory infections.

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

We will continue to publish our work in peer reviewed open access journals which will enable our work to be accessed by the wider scientific community. We have close connections with pharmaceutical companies and thus any promising therapeutic approaches would be visible to these partners.

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.

Mice are a suitable model for this project as their immune response has close similarities to humans when challenged by many pathogens and there are many well established models and relevant genetically modified mice. Mice have a long track record in being used to test pharmacological agents in the management of infectious disease so extensive pharmacological data exists for compounds which can modulate inflammation and can be

used both in murine models and in humans. There are a wide array of reagents available suitable for use in mice or mouse tissues. We have extensive experience in murine models of infection and the techniques proposed in this study. We will mainly use adult mice as they have a mature immune system similar to that seen in man. As mice have a relative short reproductive time they represent a good model to investigate inherited susceptibility to infectious diseases.

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

For many experiments mice will be infected with bacteria or viruses, such as non-typeable Haemophilus influenzae, Streptococcus pneumoniae or influenza A virus, into the lung via the nose under inhaled anaesthesia. The mice will then be killed 24 hours post infection and blood and organs removed. Their organs and cells can be examined for markers of inflammation and the amount of bacteria or virus in blood and lungs can be measured. In some experiments the mice will be kept alive longer, either for up to three weeks to study progression of the infection or several months to study the long term effect of infection. Some mice that have been infected will be bred to see the effect infecting mice has on their offspring.

In some experiments before bacteria or viruses are administered mice will be given substances which induce the features of a chronic disease, such as administration of LPS and elastase into the lung via the nose once a week for four weeks under inhaled anaesthesia which results in similar changes in the lung as seen in chronic obstructive pulmonary disease.

To investigate the effect of therapies the mice may be treated with compounds that modulate the immune response. These therapies may be administered by nebulisation into the lung or given orally in food or drinking water once or twice a day. In some cases it may be necessary to give the drug by injection, for example into the abdominal cavity. The way of administration will depend on the compound and the method chosen will be the one that causes the least amount of distress to the mouse and is most clinically relevant.

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

Mice that have been exposed to stimuli that cause inflammation may experience mild pain and/or malaise leading to reduced activity and reduced food and drink consumption which lasts less than 24 hours. Mice challenged with live pathogens may display altered mobility, reduced food and drink consumption which may lead to weight loss, and changes in their breathing dependent on the type and dose of pathogen administered. In many cases this will be temporary and the mice will regain normal activity and growth. The duration of weight loss is usually less than five days.

Mice exposed to reduced oxygen levels may display temporary reduced activity and reduced food and drink consumption for approximately 24 hrs and during this period weight loss may be observed. After the animals have become used to the lower oxygen levels they will regain normal activity and growth.

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 and 50% 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?

Animals are needed as the complex immune interactions we are investigating cannot be modelled with in-vitro systems. Although non protected species can be used to model some aspects of the immune response the important differences in physiology between them and mammals limit their use.

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

Alternatives that we have considered include the use of co-culture tissue culture systems, organ-on-a- chip systems, non-protected species (such as wax moth larvae), human challenge, and computer modelling. These approaches have been identified by literature searches, from presentations at meetings and conferences, and websites such the NC3Rs and FRAME. The NC3Rs website has provided a range of information of resources to find alternatives such as Guidance on searching for alternatives from the EURL ECVAM “Good search practice on animal alternatives”. Of relevance the EURL ECVAM has produced the “Review of non-animal models in biomedical research - Respiratory tract diseases” which covers many models relevant to the programme of work in this license. They The NC3Rs website has also been used to identify resources for the use of human tissues and in vitro, in silico and ex vivo systems.

When looking for alternatives search terms used are "in vitro" OR "in silico" OR "animal alternative*". When looking for chronic diseases terms related to the specific disease are used, such as “COPD” OR “chronic obstructive pulmonary disease” OR “Emphysema” OR “chronic bronchitis”. For respiratory infections. When looking for lung specific information "respirat*" OR "lung" OR “airway*” OR “alveol*” will be used and when looking for information about infection “infection*” OR “bacteria*” OR “vir* OR fung*”.

Why were they not suitable?

The in vitro systems are used to address questions involving single cell types or a small number of number of cells, but in vivo there are many different cell types that are found together which interact and communicate with each other in ways that are not possible to fully replicate in vitro. Similarly, non- protected species, such as wax moth larvae or embryonic zebrafish, can be used to model some aspects of the immune response but there are important differences in physiology between them and mammals that limit their use. We cannot use human challenge models to answer many of the questions such as those relating to inheritability or those that potentially result in severe 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 that will be used will vary depending on the system being investigated. For example, the variation in the number of viable bacteria measured in lungs from infected mice will be dependent on the strain of bacteria being used, the dose administered and the time after challenge. Using data from our previous work and from the literature we would expect to have group sizes of between 6 and 12 mice. We would expect that each set of experiments requires approximately 200 mice and we will perform up to five such studies a year. We will seek statistical input from a biostatistician when required.

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

We will use appropriate study design and will ensure that appropriate sample sizes are used to allow for statistical analysis and that randomisation of treatments, age and sex matching is carefully undertaken to aid reproducibility, using 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 ensure that the maximum information from each mouse is obtained without compromising the quality of the data and samples obtained. When possible we will use methods that allow for repeated measurements. Breeding of genetically modified mice will be carefully monitored to minimise breeding of mice that cannot be used but if excess unmatched stock are bred tissues, such as bone marrow, will be taken and frozen for future use. All genetically modified mouse stocks will be cryopreserved so breeding can be halted when the studies using them have finished

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 number of different methods and models in our project. The main ones are respiratory infection models where mice receive a challenge with bacteria, viruses, or fungi via the nose. This models pneumonia in humans and is a suitable approach to measure host responses to infection. In some cases mice are pre-treated to induce a chronic disease, for example COPD, and then infected with pathogens.

The dose of pathogen, the route of delivery and the length of the procedures will be chosen so that the minimum impact on animal welfare occurs for each objective. The anaesthetics that are used will be chosen to allow animals to return to their normal behaviour in a short as time as possible.

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

Species that are less sentient do not have the same immune cells seen in humans and we can’t use embryos or very young animals as their immune system is immature and the immune cells do not respond to stimulation in the way mature animals do.

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

Animals will observed at frequency suitable for the study being carried out to ensure that any adverse effects are identified promptly and addressed as appropriate. If any procedure that might be associated with pain is carried out the animals will be given suitable analgesia before the procedure and, if necessary, suitable times afterwards to minimise any discomfort. Animals will be provided with environmental enrichment, including nesting material and shelters.

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

We will use available resources including guidance and publications from the NC3Rs and Laboratory Animal Science Association.

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

We will keep track and implement advances in the subject area and technological advances that might be used to minimise harms and improve welfare and will regularly check information on NC3Rs website.

21. The role of the microbiota in poultry

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
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

microbiota, chicken, nutrition, microbial transplants, health

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	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?

To understand the interactions between the chicken gut microbiota, environment and the host bird.

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?

Microbial flora (microbiota) play a vital role in the health, nutrition and wellbeing of their animal hosts. In chickens these microbes play an important role in metabolising compounds from the chicken's feed, thereby contributing to chicken nutrition. The microbiota also has direct and indirect effects on chicken welfare, health and disease resistance. By understanding how members of the microbiota interact with the host, and

the host's environment (including diet), we can develop treatments to improve poultry productivity, welfare, nutrition and health.

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

This project will generate new knowledge on how the microbiota interacts with its chicken host and the environment (e.g. diet). It will lead to peer reviewed publications in this research area, as well as providing knowledge that can be used to design new methods to manipulate the chicken gut microbiota to improve host nutrition, wellbeing and health.

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

In the short term, this project will benefit researchers in the poultry and microbiota research fields, as it will generate fundamental knowledge about the interactions between host, microbes and diet. In the longer term, the development of interventions to manipulate the chicken microbiota could lead to improvements in the productivity, health and wellbeing of poultry; reduced antimicrobial use; as well as improving the sustainability of poultry farming. This would benefit both poultry farmers and the general public, by helping ensure that poultry products are sustainable and produced in a high welfare environment.

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

We will collaborate with a range of poultry and nutritional researchers. We will disseminate our findings by the publication of peer-reviewed articles in high quality journals, and through presentations at symposia/conferences.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 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.

In 2020 the global population of chickens was over 33 billion, with global poultry meat production increasing from 9 to 122 million tonnes between 1961 and 2017. This is expected to rise further due to population and income growth, with global poultry meat production expected to rise by 17.8% over the next ten years. The human population is also expected to increase by 2 billion over the next thirty years. It is therefore vital that we ensure that poultry farming is sustainable, enabling us to feed more people using less resources. Consumers are also increasingly aware of animal welfare in farming, demanding higher welfare products. Manipulating the gut microbiota is a valid strategy for achieving these goals. The life stages used in this project reflect those of chickens kept on farms as either broiler or layer birds.

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

Typically, experiments will begin at day of hatch and will last 5-10 weeks, after which the birds will be killed via a schedule 1 method. Our project will contain three categories of procedures: the collection of host blood/serum and gastrointestinal samples, manipulation of the gut microbiota using microbial transplants of non-pathogenic organisms, and

changing the composition of the normal chicken diet to assess the impact of these changes on the microbiota and host. Serum, blood and gastrointestinal samples may be collected at various intervals throughout the experiment, although gastrointestinal samples will more frequently be collected port-mortem. Microbial transplants are most likely to be performed within two days of hatch, but may also be performed at later stages of the experiment. If transplants are performed in older birds, they may be preceded by antimicrobial administration to decrease the abundance of the resident gut microbiota and thereby increase the chances of transplantation success. Diets that differ from standard chicken diets may be supplied for the entire lifetime of the bird, or may be provided only for a designated time.

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

When collecting blood/serum samples via a peripheral vein, or collecting gastrointestinal samples via cloacal swab, birds will experience mild and transient pain and discomfort. When conducting microbial transplants, birds will experience mild and transient discomfort during the administration of the microbes via oral gavage, spray, or intracloacal inoculation. Animals consuming diets which may not meet all of their nutritional requirements (eg. replicating a smallholder poultry scavenging based diet) or that have anti-nutritive components (eg. high levels of fibre) may experience distress caused by increased hunger and fail to gain body weight at the expected rate. Increased hunger may lead to changes in behaviour, such as increased aggression.

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:

- undergoing microbial transplantations: 100% mild.

- undergoing blood/serum sampling: 100% mild.

- consuming diets that do not meet their nutritional requirements or contain anti-nutritive factors: 100% moderate - the birds may experience prolonged discomfort.

In total, 375 chickens are expected to experience mild severity and 375 chickens are expected 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?

As this project will study the relationship between the microbiota, chicken host and diet, there is no realistic alternative to using animals. These interactions are highly complex, and could not be accurately modelled using an in vitro or in silico system.

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

- Organoids
- Batch culturing of microbes with components of the chicken diet
- Computational modelling

Why were they not suitable?

Organoids: Currently chicken gut organoids require an aerobic (with oxygen) environment, which therefore precludes their co-culture with most members of the gut microbiota, which are killed by exposure to oxygen. If the technology to use chicken gut organoids without oxygen is developed, then some questions about host-microbiota interactions may be answered using this model, and we would use it rather than live animals.

Batch culturing of microbes with components of the chicken diet: For simple questions relating to the interaction of specific, culturable members of the microbiota with dietary components, it would be possible to use microbial batch culturing. However, this does not allow us to study the impact of these interactions on the host animal. Also, many members of the chicken microbiota have not as yet been successfully cultured.

Computational modelling: While software does exist to model metabolic interactions within microbial communities, these models are not yet sufficiently advanced to be able to accurately model complex host-microbiota-diet interactions.

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 have been calculated using data from our previous studies examining the chicken microbiota, or from public datasets if it is more applicable to the study goals. Where such data is not available, pilot studies will be conducted, the data from which will be used to calculate appropriate group sizes. Previous calculations typically show that we require group sizes of five to detect changes in the microbiota relating to diet, but larger groups may be needed where previous findings have demonstrated that the treatment can lead to increased variability in the microbiota composition. Control groups have been included in our animal number estimations for all treatments, as these are essential to ensure that robust scientific conclusions can be drawn. We have demonstrated our ability to draw robust conclusions from the use of chickens in experiments, as evidenced by the publication of our previous findings in peer-reviewed journals.

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 followed the NC3Rs' experimental design guidance as well as guidance provided via our attendance at ScotPIL training courses. We have followed well established methods for statistical analysis of microbiota data, and consulted with a statistician where there were any queries on the appropriate sample size calculations or statistical methods to use.

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 data does not already exist in the public domain, we will conduct pilot studies to generate such data prior to conducting larger studies. We will archive samples to ensure that these can be used in the future, without having to use more animals. We will inform colleagues about planned studies, to allow for sharing of tissues that would otherwise not be used as part of our investigations, or that were produced in excess of what was needed for our study. Each study conducted under this licence will be scrutinised by our local AWERB to ensure that the 3R principles have been considered, prior to consent being granted.

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 vast majority of host samples will be taken post-mortem: for example, tissue samples and caecal contents. Collecting contents directly from the gastrointestinal tract allows for the most accurate characterisation of the host microbiota. However, in some studies we will also need to collect longitudinal data on host and microbiota responses. For the collection of these host samples, two methods will be used: blood/plasma sampling and the collection of caecal contents via swabbing. Both techniques are minimally invasive, and generally tolerated well by birds.

Blood/plasma sampling will allow us to monitor host responses to diet change or microbial transplantation, while the collection of caecal swabs will allow us to study their impact on the gut microbiota composition.

For the delivery of microbial transplants of commensal organisms, several methods may be used: including oral gavage, spray or intracloacal inoculation. We expect to predominantly use the former two methods, with intracloacal inoculation likely to only be used when it is necessary for the lower gastrointestinal tract to be exposed to the transplanted microbes prior to the upper gastrointestinal tract. Oral gavage and coarse spray are commonly used techniques for providing microbial transplants to chickens, with spray delivery being the most commonly used method in farms. We have previously used both techniques to deliver transplants, and this was well tolerated by the birds. Intracloacal

inoculation is a less commonly used method, but has previously been described as well tolerated).

Microbial transplants may include gastrointestinal contents from a donor bird, microbes cultured from the microbiota, probiotics or fermentation products generated from the microbiota. All of these transplant types are used extensively in research in chickens, with the latter two types also used extensively in farms, with little to no adverse effects on birds reported.

For the provision of diets that do not meet the nutritional requirements of the bird, or that contain anti- nutritive components: We will assess the impact of the microbiota on the chicken host's ability to extract energy and nutrients from non-ideal diets. This will include two forms of diet:

- diets containing substances that are found in feed ingredients but are viewed as anti-nutritive when included above a particular level (eg. fibrous compounds).

- diets that do not fully meet the chicken's nutritional requirements (eg. replicating a smallholder poultry scavenging based diet)

Such diets have been chosen as they represent dietary challenges that are encountered by the poultry industry or smallholder farmers. These diets may lead to reduced body weight gain, increased hunger and changes in behaviour due to hunger (eg. increased aggression). We will formulate the diets under the advice of a poultry nutritionist, to allow us to observe an impact on phenotype without causing excessive harm or distress to birds.

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

This project will study the relationship between the microbiota, chicken host, diet and environment. As the microbiota is host specific and the host responses to the microbiota are likely to be species specific, there is no possibility of using a less sentient animal to model these interactions. As this project examines the effect of diet and environment, there is also no possibility of using animals at a more immature life-stage (ie. pre-hatch).

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

Animals will be carefully monitored by staff who have substantial experience working with poultry and are able to recognise signs of distress and ill health. Birds will be monitored for adverse effects of study diets, such as reduced weight gain, and endpoints will be defined that ensure animals do not develop severe phenotypes. While we have not previously observed infections in chickens caused by microbial transplants, if the donor sample contains a pathogen this is a possibility. Birds who have received transplants will thereby be observed for signs of clinical illness (eg. diarrhoea, changes in mobility), birds showing signs of clinical illness will be humanely killed.

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

We will follow ARRIVE, PREPARE and NC3Rs guidelines for designing animal studies.

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

We will regularly check information on the NC3Rs website, and attend 3Rs symposia. We will attend Responsible Research in Practice webinars.

22. Role of SHOC2 in tumorigenesis 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

Cancer, Skin, Inflammation, Therapy, Cell signalling

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

The project aims to study the role of SHOC2 as a therapeutic target in cancers with RAS mutations and study what possible toxicities SHOC2 inhibition in the clinic may have and how these can be minimized.

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 cells are composed of intricate communication or signalling networks. Inappropriate activation of some of these networks is known to occur in most human cancers. The RAS-ERK (MAPK) signalling pathway is one of the most commonly affected signalling networks in human cancer.

However, studies have shown that inhibition of this pathway results in significant side effects and toxicity. The key problem is that normal cells also use the MAPK pathway to carry out their many unique functions. Therefore, drugs that target this pathway affect the

normal function of many other cells in addition to cancer cells, resulting in unwanted toxicity.

A protein called SHOC2 may provide an attractive therapeutic target to allow inhibition of the ERK pathway with the potential to overcome at least some associated toxicity. This study aims to gain knowledge on the role of SHOC2 in the initiation, maintenance and spread of different cancer types caused by mutant RAS, using mouse models that closely mirror the human disease. It will also asses the role of SHOC2 in normal tissue function and thus help anticipate any toxicities that may arise from pharmacological inhibition of SHOC2 in the clinic.

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

This project will result in important new information for the research field and will lead to peer-reviewed publications. In addition, our findings will be of the utmost relevance for the use of SHOC2 inhibitors in the clinic.

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

There is now an emerging consensus that SHOC2 provides an excellent therapeutic target against RAS-driven tumours and development of SHOC2 inhibitors is currently the focus of intense efforts by the pharmaceutical community. However key unanswered questions remain, such as what effects SHOC2 inhibition will have when studied in more complex cancer models that more closely recapitulate the human disease, as well as what toxicities may arise (and how to deal with them) after whole-body SHOC2 inhibition. This project will derive critical information on the consequences of SHOC2 inhibition, both in the context of cancer as well as normal tissue function and thus will inform and guide future treatment strategies once SHOC2 inhibitors become available.

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

We are collaborating with different research groups to maximise the output of this work, taking advantage of their different expertise.

We will also be presenting data obtained from this work at scientific meetings and we will aim to publish it in open-access journals.

Species and numbers of animals expected to be used

- Mice: ~10000 mice in 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 have opted to use adult, juvenile, and new-born mice. Mice are a well-described model organism for cancer research.

To characterise the ablation of Shoc2 at the organism level, we need to study the different life stages of the animals.

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

In general, animals will experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal) or by oral dosing. In cancer models, we may induce tumours genetically or by injection of cancer cell lines.

Studies will usually not last longer than 1-2 months.

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

Animals on tumour protocols are expected to develop cancer within the boundaries of this project and approximately half will receive drug treatments designed to inhibit the progression of these tumours and/or regress larger established tumours.

The majority of animals (up to 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, for example animals on lung tumour protocols, may develop breathing difficulties. In addition, certain genetically altered mice may present with lesions of the skin, inflammation and weight loss. 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)?

Based on our vast experience using these procedures and experimental models we anticipate about 85% to 90% of mice to experience Moderate severity and 10 to 15% Mild. 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) injections of therapeutic agents or oral delivery. We will aim to utilise the least stressful route of administration wherever possible.

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 is not isolated from the host in the clinic and instead therapeutic efforts to target cancers must take into account cancer as part of the patient. This leads to a trade-off of therapeutic efficacy (desired effect) versus possible toxicities associated with any therapy,

as well as pharmacodynamics (the ability of the drug to reach its site of efficacy ie. the tumour without being broken down and excreted by the body). Culture methods cannot consider these complications, and as such mouse models must be used to better reflect treatment options in a whole organism. Similarly cancer has been shown to be both impeded by, and conversely, manipulate host systems including the immune system. Therefore when designing the next generation of anti-cancer agents the cross-talk between cancer and non- cancerous tissues in an organism must be taken into account as they can influence both efficacy and toxicity.

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

Cell culture based systems have been thoroughly considered, alongside published literature. Patient- derived tissues and explants have also been considered.

Why were they not suitable?

We have thoroughly investigated our scientific aims using human and mouse cell lines. We now require systems that fully recapitulate the complex interplay between cancers and the immune system which is only possible using a whole organism 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 taken into account the estimated size of experimental cohorts for each different protocol we will perform including pilot experiments, how often we anticipate performing them, and the overall importance of each protocol to our scientific aims.

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

Group sizes will be kept to the minimum required to obtain statistically sound data. In the case of uncertainty of the experimental outcome, pilot studies are proposed to determine whether the potential gains in terms of useful data merit the animal usage. Colony sizes will be carefully managed to ensure that supply matches demand. Whenever possible, surplus mice/tissues will be used for other scientific purposes. In some cases, where a particular strain of mouse is not needed for long periods of time, mouse embryos of a particular strain will be kept as frozen stocks thus keeping mouse numbers to 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?

Where possible pilot and/ or optimisation experiments will be performed in tissue culture. We aim to derive mouse tumours from animal and perform many experiments on these cells in vitro, limiting overall mouse numbers.

At the end of the experiment, we aim to derive mouse tumours and perform many experiments on these cells in vitro, limiting overall mouse numbers. We will also harvest as many tissues as possible at post-mortem for further characterization.

We are using the most efficient breeding strategy to minimise the number of animals used to obtain the experimental 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.

We will use various mouse models of conditional deletion of Shoc2 and other genes, therefore mice will not exhibit any medical symptoms until they are undergoing an experiment and their genes are deleted.

To delete gene expression or to deplete specific cells, some animals will be given substances by oral gavage, injection, or through food.

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

Mice are a well characterised model for cancer studies that can recapitulate the effects of the immune system and cancer in the whole organism. Less sentient animals are not as well-characterised and would not allow us to properly study the effects of Shoc2 ablation in the whole organism. In addition, mice are the lowest vertebrate animals that have been shown to closely mimic human disease and therefore provide more relevant information.

Non-mammalian animals are limited in their use because they either do not have the right type of immune cell or their immune system is too different from the human immune system to provide relevant results.

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

We have access to experimental conditions used to help us to design our experiments to minimise animal usage, minimise the length of the experiments and perform the most appropriate types of analysis. These models employ minimally invasive techniques and can be studied using non-invasive imaging. Whenever possible, we will use the minimum volume for all dosing routes for each protocol.

When using existing chemical inhibitors, usage concentrations, in terms of effectiveness and tolerance, have already been tested by other parties, eliminating the requirement for us to repeat these experiments. In addition, where possible drug will be given in the diet of the animals, negating the need for alternative dosing methods.

We will monitor the animals very closely and we will try ameliorate the suffering if observed. We will seek advice from the NACWO and NVS at all times to ensure the mice wellbeing.

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 guidance and publications from the NC3Rs and Laboratory Animal Science Association.

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

We will stay up to date about advances in the 3Rs by communicating with the institutional representatives of the 3Rs at UCL. We will regularly check information on NC3Rs website and to implement these advances we will refer to the NC3Rs guidance at all times.

23. Developing novel combination therapies 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
- 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

Cancer, Combination therapy, Radiotherapy, Colloidal delivery systems, Formulations

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 develop new, more efficacious and less toxic treatments for cancer using combinations of drugs, nanoparticles and radiotherapy in various novel and oral formulations that we will develop. These treatments, when combined together in the right formulations and in the right order, will cause cancer cell death, but will cause less harm to normal non-cancerous parts of the body and should cause less side effects.

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 the numbers of people in the UK getting a cancer diagnosis has stabilised, the fact remains that one in two individuals will face a cancer diagnosis in their lifetime.

Despite recent advancements in treating certain cancers like testicular, lymphoma, and breast cancer, mortality rates persist at high levels. Additionally, many existing treatments come with significant side effects due to their impact on healthy cells. There is an urgent need for new treatments that are more effective in targeting cancer cells while minimising harm to healthy tissues.

Currently, radiotherapy is employed in treating approximately 40% of cancer patients, but its usefulness could be greatly improved by delivering radiation precisely to tumour sites while sparing normal cells. This targeted approach holds the potential to enhance tumour eradication without causing debilitating side effects.

Targeting can be achieved both by using different types of radiation but also by making the cancer more sensitive to the effect of radiation by using therapies called radiosensitisers.

These radiosensitisers can either be drugs or genetic therapies or nanoparticles but the challenge is to selectively radiosensitise only cancer cells.

The proposed studies aim to develop novel treatments by combining drugs or nanoparticles and various types of radiation to specifically target and eliminate cancer cells while preserving healthy tissues. This approach, already showing promise in producing less toxic cancer treatments, could significantly improve patient outcomes and reduce treatment-related complications. Moreover, findings from laboratory work and animal studies conducted previously in our laboratory have already informed clinical treatment strategies, and further research will guide the development of even better therapies for cancer patients in the future.

Furthermore, the use of common, more affordable drugs in the treatment regimens may lead to cost- effective treatment options, benefiting both healthcare providers and patients.

Additionally, the innovative approach to radiation, drug and nanoparticle administration may contribute valuable insights to basic scientific knowledge, informing future research and treatment development efforts. Ultimately, the knowledge gained from these studies has the potential to advance cancer treatment practices, benefiting patients, healthcare providers, and the broader scientific community. Furthermore, this knowledge gained from these proposed studies is very important for cancer research scientists as we can make more effective treatments. If we understand how various combination therapies affects cells, we can understand why some treatments don’t cure cancer and improve upon them.

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

At the end of this project, we hope to have developed novel combination therapies for hard-to-treat cancers. We hope to have demonstrated that optimal combinations of drugs or nanoparticles and or radiation, in the best formulations if given in the correct sequence, can cause tumour regression in mouse models of hard-to-treat cancers such as brain tumours, triple negative breast cancer, soft tissue sarcoma, pancreatic cancer, colorectal cancer and childhood cancers. We hope to have developed a scientifically rigorous and convincing pre-clinical package to entice our medical partners and clinical trial sponsors to work with us to translate the best formulations into the clinic to improve survival and reduce toxicity in cancer patients.

We will have added to the knowledge base about how these cancers can be treated, what works and why. We will publish and share our findings to enable other researchers to be

able to use this knowledge to progress their cancer research and not to repeat experiments that do not work as well in animal models and in vitro studies.

Specifically, the data will add to the understanding of the basic biology of how various DNA damage and repair pathways function and can be abrogated in cancer cells in vivo or how the addition of genetic material can lead to cancer cell death.

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

Outputs from the project will be used by our own group, our collaborators and when published and disseminated, will inform others in the radiation oncology, cancer research and biology community.

If successful, the outputs from the project will form the basis of a pre-clinical package which will be utilised to attract clinical collaborators and clinical trial sponsors to translate our work into clinical practice. We have already achieved this aim with previous projects under our previous licences.

We hope that ultimately, benefits will be gained to cancer patients and health care providers and funders. We hope to discover more effective cancer treatments designed in such a way as to limit the dreadful side effects most cancer patients currently suffer with many treatments and to do this using affordable drugs that are potentially repurposed drugs thus reducing the time and cost to clinical application.

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

The outputs from this project will be maximised primarily through dissemination of knowledge via open access peer reviewed publication. Our previous research by the Licence applicant has resulted in 117 publications and they are very experienced in converting scientific findings into publications. Work will also be disseminated through presentation at conferences and via our extensive network of clinical and Industry collaborators and via internal mechanisms such as our Institutional research groups and strategic themes and Initiatives.

Knowledge Transfer is critical to disseminate positive findings but also to feed forward and backwards unsuccessful findings as well as methodology development that can reduce, replace and refine. This will occur through events such as our Institutional Open day, NC3Rs meetings, Engage events and through social media where both the applicant and the institution are very active.

Our group by its nature is multidisciplinary with a wide range of home and international collaborators in other cancer and biological disciplines and well as with collaborators across the physical sciences. All of this experience and different perspectives will be brought to the planning and execution of our proposed experiments and in the process results, techniques and the principles of the 3Rs will be disseminated so that others in these disciplines become familiar with the optimal way to design and execute in vivo experiments to optimise outputs with animal welfare at the heart of experimental design.

Species and numbers of animals expected to be used.

- Mice: 2000
- Rats: 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.

Cancer models using cell lines or co-cultures are useful for pilot studies but tend to be limited in their reproducibility of clinical disease due to not fully addressing the complexity of cancer development and cell to cell interactions. Rodent models offer data that is comparable to the human disease, due to their complex biological systems which cannot be found in less sentient species and are the most commonly used species for cancer work. Mice are utilised in the major protocol in this study as they are the lowest vertebrate group which are well characterised for the establishment of models that are accepted for cancer experimentation.

Rats will be used only to undertake drug delivery or pharmacokinetic experiments using oral drug delivery systems (e.g. Oral Thin Films (OTFs), mixing with food/water; or oral gavage). This is because larger rodents are required both to administer the film (OTF), which due to diametric and formulation specific issues cannot be made smaller to enable fit in the mouth of a mouse without significant risk of harm to the animal, but also to allow withdrawal of enough blood for biodistribution studies. Adult animals will be used in all protocols.

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

Tumours are introduced either subcutaneously (cancer cells introduced under the skin to form tumours on the flank called xenografts), or by intravenous (in the vein), intraperitoneal (in the abdominal cavity) injection of tumour cells or bioluminescent tumour cells (which express the luciferase transgene which make the cells fluorescent to be able to be visualised) to create metastatic models of tumour growth.
Metastatic cancer models are where cancer cells are injected into the mouse so that the cancer spreads throughout the body to mimic what happens when a persons cancer spreads.

Xenograft growth is measured by physical measurement of the tumour growth over time using callipers. For metastatic models it is not possible to undertake a physical measurement as the tumour is internal. Instead, bioluminescent cell growth will be measured on a bioluminescent imager (BLI) using established parameters for correlation of bioluminescent output and tumour size. The parameters for measurement of tumour size in these models will be validated in a small number of tumour-bearing animals post-mortem. This mode of measurement will then be deployed to allow serial analysis in a single mouse and thus utilisation of fewer mice overall. Other non-invasive imaging modalities (e.g., CT) may be used in some instances in order to gain more information on tumour growth/spread.

Standard checklist parameters such as weight and health status will be employed to gauge animal wellbeing simultaneously with specialist imaging that allows us to view the tumour internally.

For dose-range-finding experiments, mice are given escalating doses of agents.

While this leads to more interventions and the potential for more adverse effects, we believe that these are minimal and the experimental design will allow significant reduction in overall mouse numbers required.

For experimental treatments, animals will be given treatments applied to the cancer cells in a singular form or a combination of the following:

- Radiopharmaceuticals
- Chemotherapy or non-chemotherapy drugs (repurposed drugs) which target signalling or repair pathways which render cancer cells more sensitive to radiation
- Metallic or lipidic nanoparticles
- External beam irradiation
- Plasmids or viruses which express sequences enabling direct targeting of pathways described in 2 above
- Viruses which act as gene delivery vehicles to introduce transgenes which enhance tumour cell kill;
- e.g. genes which encode transporters for radiopharmaceutical uptake
- Drugs which enhance or enable tumour growth such as oestrogens
- Delivery vehicles such as oral thin films or novel formulations
- Experimental agents will be delivered via oral (mixed in food or water or by oral gavage, pipette or as an OTF), intravenous, intraperitoneal, intratumoural (only in subcutaneous tumours) delivery routes using minimal quantities possible and as per local dosing guidelines.

Body fluid sampling (blood/urine) will occasionally be undertaken only if required to measure drug/radiopharmaceutical/nanoparticle concentrations and tissue may be collected post-mortem to allow molecular interrogation or measurement of dose limiting organ toxicity. Occasionally, if larger quantities of blood are required for experimental purposes such as PK analysis, a non-schedule 1 method will be undertaken (i.e., exsanguination by cardiac puncture under deep terminal anaesthesia).

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

In some experiments we need to use short-acting anaesthetics to make the animals sleep during the experiments. Although inducing anaesthesia is always a bit stressful for the animals, this is to reduce any short-term pain they may experience by those procedures.

In some cases, cells or drugs or agents will be injected into the animal which may cause a transient discomfort but, again, by using only well trained and experienced people, together with analgesics when appropriate, we will minimise this adverse effect and the volumes and routes of administration are chosen to cause the least possible discomfort.

The growth of the tumour on the flank of the mice after injection of cancer cells does not cause the animal any overt adverse effects. Mice who have metastatic cancer may become unwell; however, these experiments will be stopped before the induced cancer causes major harm, and the wellbeing of the animal will be monitored carefully and very regularly. Any animals that show signs of illness, weight loss or lack of general health and wellbeing will be euthanised. Furthermore, any metastatic models will be trialled in a small number of animals (pilot group) so we know exactly what the course of the disease will be and can ensure animals are euthanised well before they experience unacceptable adverse events.

The drugs and agents we will be giving to the animals are mostly well characterised and have been used in other experiments at doses that are safe and are reported in the literature. Nevertheless, it is possible that some agents may cause adverse effects.

Therefore, any new drugs or agents alone or in combinations will be tested first in a small number of mice using a protocol called dose-range finding studies which consists of using only one mouse at a time. This approach lets us try a few doses based on the levels of similar drugs in the literature to ensure they cause no or minimal harm before giving the drug or combinations to a larger group in the therapeutic studies. If any drugs or combinations cause unacceptable weight loss, ill health or adverse effects they shall not be used again.

Occasionally, animals will be restrained during procedures. Only well accepted methods of habituation and restraint will be used so as to minimise stress. Most animals quickly become used to the restraint, but if there are signs of distress the procedure will be stopped.

The effects of radiation and radiolabelled drugs on animals are very well documented and through our own previous 25 years of work and other published studies, we know that the radiation causes minimal adverse effects on the animals (slight and temporary lassitude).

If any animals show signs of ill health, they will be withdrawn from the study and euthanised.

Blood samples may be taken from the animals; this may potentially cause anaemia. To avoid this, no more than 10% of the animals' blood volume will be withdrawn on any one occasion and the guidelines for blood sampling will be followed.

Most of the mice used in our studies have been bred to have a defective immune system as this is the only way we can grow human cancer cells in the mice, so their bodies will not reject the cancer. The animals are kept in sterile housing with sterile food and water to avoid the mice being exposed to possible infection and any procedures will be similarly done in a sterile environment to avoid infections.

Occasionally, we may need to withdraw food and drink from the animals, but this will be transient and again will adhere to guidelines. The animals may be kept in special cages that allow us to collect faeces and/or urine but only for a minimum period of time and with no expected adverse effects.

For animals who are being given oral thin films on their tongue there is a small possibility of irritation or discomfort in the mouth. This risk is limited by the animals being anesthetised before we apply the film and the animals being monitored closely after application for any signs of 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)?

Both protocols are expected to have, at most, a moderate severity limit but this is really the worst-case scenario and actually most of the experiments are expected to have a mild

severity. So, for both mice and rats we expect 80% of animals to experience a Mild severity and up to 20% possibly 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?

The use of live animals is required to:
Examine the impact on tumour growth delay of combinations of therapeutic schemes and formulations;
Model the distribution of drug, nanoparticle or radiopharmaceutical uptake from the bloodstream;

Assess the potency, pharmacokinetics and biodistribution and mode of action of multiple tumouricidal agents.

Furthermore, to enable clinical translation, pre-clinical studies are required to confirm or refute in vitro data.

For the majority of trials, in vivo experimentation is the last resort and will only be performed when extensive in vitro experimentation has been undertaken. For this we have developed in vitro 2D cell culture models using algorithms to determine optimal combinations (median effect principles and Combination Index analysis- (Chou, 2010)) and 3D tumour spheroids models which we have optimised in terms of creation of larger spheroids using new technology (McMillan *et al,* 2016) which will enable analysis of combinations in models which are far more representative of tumours in vivo than 2D models. We are experts with this system and have identified that the outcomes from spheroid experiments are far more predictive of how novel combination therapies will behave in vivo.

Since our last project licence, we have secured an NC3Rs Technological Transfer Grant and were assigned a significant space to develop a Chick Embryo Tumour model. This model uses animals of much lower sentience than adult rodents and is considered a partial replacement. It offers a further step in our pre-murine model cascade, that allows the consideration of tumour growth and cell kill in the presence of pumping blood vessels and allows biodistribution and toxicity studies that can significantly reduce the number of murine models we use, as we can rule our inferior therapy combinations which would previously have been tested in murine cancer models. This in vitro cascade and the development of models, techniques and their application will allow us to replace animals for this stage of the experimentation and utilise our in vivo models only for already validated successful combination schemes.

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

We have undertaken a comprehensive literature search using online databases from

around the world as well as patent search databases and have considered relevant websites such as NC3R and NC3Rs Oncology Network and FRAME, to identify alternatives to the use of animals in this specific research project. While there are some models under development that may be ready and useful in the future it is too early in their development to consider their use instead of animals.

We already use 3D tumour models derived from human cancer cells and as described above have through NC3Rs funding, developed a chick embryo tumour model.

We have also been utilising online genomic data and the human protein atlas to undertake non- laboratory experiments to research drug combinations and effects on their targets, as well as which specific tumour cell lines with particular mutations we should be utilising for our in vitro and then in vivo experiments, enhancing our chances of success and reducing trial and error experimentation.

Why were they not suitable?

The use of in vitro models such as multicellular spheroids, can provide information on the efficacy of various therapeutic schemes or formulations at a simplistic level and inform on the best formulations and what to take forward to in vivo interrogation, but does not allow the assessment of time-dependant distribution and impact of therapeutic schemes in tumour and normal organs. Likewise, the use of the CAM model can offer more in terms of biodistribution and efficacy, but the limited time frame for tumour growth after treatment does not allow for longitudinal monitoring of tumour growth or the effect of treatments on mammalian systems and organs. These pharmacokinetic aspects of drug/treatment development can only be assessed at this time in vivo.

Until in vitro technology is developed enough to allow the same level of detail as in vivo models, they are the only suitable option as non-animal alternatives do not allow reliable predictions of the tumours response to therapy due to lacking the responses of the complex biological systems and cellular interplay seen in living animals. While there are some models under development and identified in for example the NC3Rs Oncology Network that may be ready and useful in the future, it is too early in their development to consider their use instead of animals or to reduce the number 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 are based on our previous licenses and our estimated usage over the coming 5 years. We have also contacted the Institutional named biostatistician to confirm that these numbers are suitable to achieve the goals set out in this license.

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

All experiments will be designed to utilise the minimum number of animals possible, in

consultation with the Institution's biostatistician, the NC3Rs EDA and through the website www.3Rs-reduction.co.uk

.

All studies are first extensively performed in vitro and only promising formulations are carried forward for experimentation in the (least invasive) subcutaneous xenograft models. Formulations and therapeutic schemes which show promise in xenografts will then be evaluated in systemic tumour models. The experiments are designed to allow evaluation of many treatment groups within one experiment. This limits the number of animals utilised in control groups and the data generated allows direct statistical comparison between the treatment groups and relative to the control group. The numbers of animals proposed are the minimum which will allow rigorous statistical analysis and are large enough to preclude multiple repetitions. These numbers are based upon both our previous investigations (25 years' experience, published and internal) and the advice of statisticians.

The experimental design will analyse several endpoints:
Radiopharmaceutical – measuring the percentage of the initial injected dose in tumours and organs post-mortem to assess the ratio of tumour to normal organ uptake. Measuring tumour to liver uptake will be used to assess the efficacy of the formulation. Results will be calculated by estimating the areas under the time-activity curves, correlated for radionuclide decay, multiplied by the equilibrium dose constant for the various radionuclides.

Nanoparticles with or without drugs or nucleic acid-following administration of nanoparticles at various time points: measurements such as nanoparticle concentration or gene expression or knockdown will be taken to assess either particle distribution (using BLI) or following sample collection during the experiment or post mortem.

Therapy studies – growth delay or tumour cure will be assessed by calliper measurement of xenograft size. Reduction or abrogation of metastatic tumour spread will be measured by non-invasive bioluminescent imaging. Formulations which result in statistically significant delay of tumour growth or cure or a reduction in bioluminescent intensity (metastatic spread) compared to controls will be deemed satisfactory. We will utilise bioluminescent imaging where appropriate, allowing the serial analysis of live mice, thus allowing significant reduction in animal numbers.

Range-finding studies using dose escalation – this requires a smaller number of animals compared to assays using single doses at different dose levels. Numbers are a result of experience and statistical advice.

PK studies: this experiment will be designed using a pooled animal matrix, where each time point utilises different animals' sampling so the same animal will not be utilised for blood draw and analysis at every time point. This approach has been utilised in previous experiments on an alternative animal licence successfully, with only moderate effects on the animals, and this enables extensive PK data to be collected from a smaller number of animals.

Data will be assessed using appropriate statistical parameters previously utilised and based upon the data set. Data will also be rigorously assessed at each stage and the statistician consulted between experiments to ensure that any alternation in design can be applied as the licence progresses. Sample size has been and will continue to be set using power analysis, generally the significance level will be 5%, and the power 80% but data will be assessed on a case-by-case basis through consultation with statisticians, to design the most appropriate experimental scheme.

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 purchased as needed and we will not run a colony of animals which would likely results in wastage.
Each experiment as well as preceding in vitro experiments will inform those experiments which come afterwards. In this way the use of small dose ranging studies will inform larger efficacy studies and data generated from larger experimental studies will be utilised to refine experimental design. Our programme of in vivo study will thus be iterative.

For the tumour model we use most extensively, i.e., the subcutaneous model, for the 20-30% of mice where the tumour doesn't "take", we will harvest pluripotent stem cells from the nasal passages after humane killing, which allow us to obtain glial cells for our in-vitro experiments. We will also utilise these mice where tumours have not formed to establish a tissue bank, where we will take tissues and make paraffin embedded tissue for use as an Institute research and teaching resource for immunohistochemistry. In this way, these animals are not wasted nor is there a need to use other animals to harvest these 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 athymic nude and SCID mice for the majority of studies (with the exception of PK studies), the most suitable models for establishment of the human cancer models we use. We will also, where possible, utilise wild type mice for syngeneic tumour models with respect to the scientific hypotheses and aims, limiting the need for genetically modified animals.

Wild type rats will be utilised for studies investigating the formulations or delivery of nanoparticles and drug release profiles of Oral Thin Films (OTFs) as we require a larger animal for delivery of the OTFs to the tongue (mice are not suitable given the size of the film) and also to enable repeated blood sampling from the tail vein.

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

Models using less sentient species do not share the same similarities seen between that of the rodent and human in terms of complexity of biological systems such as immune systems, organ activities, metabolism, circulatory systems etc. making it harder to study the human response with respect to drug or nucleic acid delivery and tumour growth with those types of models.

Currently the zebrafish model is the only other less sentient non embryonic model available that could be utilised for these types of studies; however, the data from this model is not

still comparable to the data that is achievable using rodent models at this current time. Likewise, we have developed a chick embryo tumour model and while we can utilise this model to replace some murine models in some settings and can also reduce the number of murine models we require, the model cannot provide the complexity and information that we require for some experiments.

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

We will be ensuring that all experiments are designed to minimise suffering and techniques used are those best for the animal. We have learned and evolved considerably over the years that we have held similar animal licences, refining at each state and within experiments in each licence.

Suffering will be minimised by:
Using all available published knowledge and experience to predict adverse effects.

Provision of appropriate enriched environments for animal maintenance.

Adherence to local guidelines and the “Guidelines for the use of animals in cancer research (2010).

Only experienced licensees will undertake procedures and continued training and monitoring will be undertaken by the licence holder and NTCO to ensure best practice.

Immuno-compromised mice will be maintained in IVCs (individually ventilated cages) under barrier conditions to avoid infection.

Anaesthesia and Analgesia will be used where it is best to do so, in consultation with the NVS.

Animals will be monitored closely before, during and especially after procedures.

Early humane endpoints will be used and these will be refined in light of experience and according to local and published guidelines. Protocols will be put in place (as we have done so previously) to monitor the condition of the animals using a scoring system to assess their health and wellbeing. Serial termination of a small number of animals will be employed when optimising new systemic tumour models, in order to detect and profile internal tumours and confirm BLI imaging for subsequent studies.

This will also allow modification of subsequent experiments to minimise animal suffering.

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

We will be using the local guidelines relating to sampling that we have collaboratively developed with other users and BPU personnel through many years of experience and dosing volumes and frequencies and will also follow those provided in the Workman *et.al.* "Guidelines for the use of animals in cancer research (2010)" and the good practice guidelines for administration of substances from the NC3Rs.

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

We are open to making changes to our work that result in refinements and strive to do so. We will be reviewing scientific articles for new models that may be of use and consider any new options that become available to us. We will also consult with the Universities 3R's champion, Named Information Officer, Named Veterinary Surgeon and Named Animal Care and Welfare Officer on any new options that they discover that could be useful in moving towards non-animal or less sentient models or any new refinements that could be made to our experiments to ensure we are putting animal welfare first.

We will also be monitoring the NC3R's outputs including the NC3Rs Oncology network for new suitable models and refinements. We will attend conferences and in fact disseminating the utility of the Chick Embryo tumour model to various networks is a long-term goal of our NC3Rs award and through these KE activities we will also be constantly seeking ways to implement the 3Rs.

24. Drug Discovery for Diseases of Poverty

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

Efficacy, Drug Discovery, Chagas Disease, Visceral Leishmaniasis, Bacterial persisters

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 and bring forward new treatments for diseases of poverty such as visceral leishmaniasis, and Chagas' disease. This project will assess the effectiveness of treatment with active substances with drug-like properties in appropriate animal models of these diseases. We would also like to start to identify new potential treatments for bacterial persistence, which is a key factor in the development of antibiotic resistance in infections. Initial pilot studies will be carried out to establish a model of bacterial persistence and assess efficacy of newly identified substances that kill bacterial persisters in invasive non-typhoidal salmonella 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?

Current drugs are unsatisfactory for a variety of reasons, including high cost; unacceptable (often serious) side effects to adults, pregnant women and children; the need to be given by injection rather than by mouth; and increasing treatment failures, often due to the

emergence of drug-resistant strains of parasite or bacteria.

The diseases we study include visceral leishmaniasis and Chagas' disease which collectively cause about 80,000 human deaths a year. We also wish to commence studies on bacterial persistence/antimicrobial resistance with a focus on Salmonella Typhimurium that causes chronic and relapsing infections with high burdens of morbidity and mortality, particularly in Low and Low-Middle-Income Countries (LMICs). Multi-drug-resistant isolates of S. Typhimurium that are prevalent in sub-Saharan Africa cause invasive disease in humans (iNTS – Invasive NonTyphoidal Salmonella) at high rates, leading to 421,000 cases and approximately 50,000 deaths per year in Africa alone, and disproportionately affecting children.

Additionally, information learned from studies against S. Typhimurium may positively impact drug discovery efforts against other forms of multi-drug resistant bacteria. A failure to address antimicrobial resistance more generally has been reported to potentially lead to around 10 million deaths per year by 2050.

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

This project seeks to discover better and safer treatments for parasitic and bacterial diseases that afflict millions of people living in tropical and sub-tropical regions of the world. The outputs include information and potential treatments for two "neglected diseases" (visceral leishmaniasis and Chagas disease) which collectively cause about 80,000 human deaths per year. We also wish to commence studies on bacterial persistence/antimicrobial resistance with a focus on S. Typhimurium that causes chronic and relapsing infections with high burdens of morbidity and mortality, particularly in LMICs (Low- and Middle-Income Countries).

The data generated in this project will be essential to the development of new medicines as they provide a means to select substances for full preclinical development and thence into clinical trials. Four new potential drug treatments have entered clinical trials from my previous licences. We expect the findings of this project to advance new candidate drugs for diseases of poverty closer toward clinical development.

We expect to publish several research articles about our discoveries concerning drug discovery for Chagas Disease, visceral leishmaniasis, and bacterial persisters.

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

In the short-to-medium term, the benefit of this programme of work shall be to fill the drug pipeline for these diseases of poverty with high quality candidate molecules with differing modes of action for progression through regulatory toxicology and entry into clinical trials. With attrition in drug development, it is critical that a number of candidate molecules are identified and progressed.

Additionally, short-term benefit of the pilot studies for investigation of bacterial persisters is the development and refinement of efficacy models relevant to these diseases to enable further drug discovery efforts in these areas.

The long-term benefit would be the delivery of new, safe, and affordable marketed drug treatments, that will have a major impact on the health and wellbeing of people.

This shall benefit healthcare providers by overcoming the problems associated with

currently available treatments, improve patient compliance and shall benefit patients by improving the outcomes of disease treatment.

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

When possible, we will share data, compounds we make or use, and methods with other researchers in the disease areas we study. All datasets and publications will be open access so that they can be accessed by other researchers and members of the public. We shall publish articles where the methods are described in detail.

We will use the information we gain, with the help of our collaborators, to develop new therapeutic treatments for Chagas disease, visceral leishmaniasis and S. Typhimurium.

Species and numbers of animals expected to be used

- Mice: 3700

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.

Previous studies into the diseases of interest have used mice, rats, and hamsters, however we will use only mice in this project. Mice have been chosen as they have predictable infectivity and pathogenesis in the currently used models and are readily available with a number of useful and informative genetic alterations. Additionally, mice have fewer species-specific metabolic differences than hamsters, and for some diseases being investigated, mice show fewer adverse effects e.g. Rag2KO immunocompromised mice (that do not develop mature T or B cells important for immunity) display fewer adverse effects than hamsters when infected with visceral leishmaniasis.

Infection with the diseases of interest affect humans across all stages of life. Adult mice are used, as adult animals typically show fewer adverse effects from acute infection than juvenile animals.

Genetically modified or spontaneous mutant animals may be used where genetically normal ‘wild-type’ animals are not suitable to provide the needed scientific outcome. For example, where in vitro drug metabolism and pharmacokinetics (DMPK) data (consideration of how a drug is metabolised and processed by the body) shows that drug metabolism in wild-type mouse cells is drastically different from that seen in human cells, to a level which prevents progression to efficacy in wild-type mice, then mice which are genetically altered for humanised metabolism (e.g. 8HUM mice who are genetically altered to produce specific human liver enzymes instead of mouse ones) may be used.

Genetically altered immunocompromised animals may be required for the production of sufficient numbers of parasites for use in other protocols.

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

Our interventions are of two types. Firstly, we shall test the efficacy of novel compounds to

treat the disease state in the rodent model systems. Of course, we shall only do this when there are robust data from laboratory studies (initially using non animal methods including cell-based tests and computer modelling, followed by small-scale pharmacokinetic studies conducted under a separate project) to suggest that the compounds are active, have suitable pharmacokinetic properties and not generally toxic. Generally, animals shall be infected with parasites or bacteria from one of the diseases of interest for this project. After infection, the level of infection shall be monitored either via the collection of blood, or via an intermittent schedule of non-invasive imaging. Where non-invasive imaging is used, each imaging session typically requires an injection of a substance which allows the parasites to be detected, and animals are maintained under anaesthesia while images are being captured (typically<20 minutes per animal). Dosing regimens will depend on the parasite or bacterial strain, and the available data for the compounds being investigated.

For efficacy studies for Chagas disease, caused by the parasite T. cruzi, single compound treatments will generally dose once or twice daily for 20 days, while combination treatments and studies for other parasites or bacteria will usually be less than this. Blood sampling for pharmacokinetic/pharmacodynamic (PK/PD) analysis will be carried out on selected animals from each experimental group (typically 10µL per sampling occasion, at 5-6 time points after the first and last administration of treatment). This allows us to measure the amount of drug present in the blood in order to: determine if drug levels in the blood change over the course of the treatment due to the body's response to the drug; check that the drug levels remain above the level likely to be effective between dose administrations (indicated by comparison with cell-based activity assays); indicate whether successful treatments have potential to be effective at a lower dose level; and to provide information to help find possible reasons for failed treatments. For some parasitic diseases, animals with apparently successful treatment (as measured by a lack of parasitic signal during non-invasive imaging) will be administered an immunosuppressive agent (a drug that reduces the effectiveness of the immune system in fighting infection e.g. cyclophosphamide) to induce relapse of any residual infection. Animals without relapse after immunosuppression (typically as indicated via non-invasive imaging) will usually be humanely killed prior to removal of organs for further analysis. Animals with unsuccessful treatment or relapse (indicated via a return in parasitic signal during non-invasive imaging) will be terminated via an approved and humane method.

Experiments investigating infectivity and pathogenesis (how the parasites cause disease) follow similar study designs to efficacy experiments, however these animals will not receive a novel treatment, but may be administered doxycycline (antibiotic) in drinking water sweetened with 5% sucrose.

Secondly, in order to have parasite preparations that are representative of those that are infectious in animals, for use in efficacy studies, we shall have to harvest parasites from infected animals. These animals shall also receive a single inoculation with parasites, and the level of infection shall be monitored via blood sampling (typically 5µL blood per sampling occasion) or via non-invasive imaging.

Where non-invasive imaging is used, each imaging session typically requires one injection of a substance which allows parasites to be detected, and animals are maintained under isoflurane general anaesthesia while images are being captured (typically <20 minutes per animal). Once the level of infection has reached the pre-determined level for the species of parasite being used, T. cruzi-infected animals will usually be exsanguinated via cardiac puncture under terminal anaesthesia and killed via a humane approved method. For visceral leishmania studies, blood is not required, so animals are killed via a humane method before infected tissues are collected.

Animals used for the production of macrophages (an important white blood cell that captures and kills microorganisms that cause infection) will receive a single injection of an inflammatory stimulus, then will be terminated within 3-7 days by a Schedule 1 method.

The macrophages will be used for in vitro analysis to investigate cellular/parasite culture.

The duration of experiments will range widely, depending on the parasite under investigation. Production of T. cruzi trypanosomes in Rag2KO mice (a genetically altered mouse strain where the immune system functions less well than in normal animals) typically require 9-10 days from initial infection to termination, whereas L. donovani parasites (which cause visceral leishmaniasis) may be collected over 50 days after initial infection when animals are infected by the intraperitoneal route.

Chronic T. cruzi efficacy studies with our standard reference strain generally last 5-6 months from initial infection to end-of-study, with treatment most commonly starting at least 100 days after initial infection in order to allow the infection to reach the chronic stage. Visceral leishmaniasis efficacy studies with our standard reference strain, L. donovani, typically last for 19 days after infection.

A robust protocol for investigation of bacterial persisters has not yet been established, so the exact study design and times required to reach the scientific endpoint are not fully defined, however they are generally expected to last less than 2 months.

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

Our rodent models of infection closely mimic the course of the human diseases and the outcome of treatment with the currently available medicines. The early symptoms of these infections are flu like (fever, loss of appetite, general malaise), but, like human patients, can be ultimately fatal if not adequately treated. By frequent monitoring of the health of infected animals, we can usually withdraw animals from the studies and kill them humanely before signs of serious illness or death from the disease occur. However, our initial studies on T. cruzi (the causative agent of Chagas disease) have demonstrated that these observations are not always predictive of the stage of disease, mainly because these parasites hide in the tissues of the body, rather than circulating in the blood. We have therefore applied advances in whole animal non-invasive imaging technology to measure the total parasite load and thereby reduced the severity of the protocol for developing new medicines for Chagas Disease.

In these studies animals may have transient discomfort from dosing the potential medicine (this is usually done by injection into a vein or by giving it via a tube passed down the throat and into the stomach). Toxicity is not generally expected, as potential treatments will already have been tested via in vitro assays, and in vivo pharmacokinetic studies. In the rare instance that a treatment is toxic the animals may lose weight, start to have muscle tremors or become very subdued. Animals showing these signs would be immediately humanely killed. If the compounds don’t work at killing the parasites, or the animals are in a group that don’t receive a treatment, they can show clinical signs of the disease as described above and will be humanely killed if the humane endpoints are reached.

Non-invasive imaging is not expected to cause adverse effects due to the short period of time under anaesthesia and infrequency of imaging. Good practice guidelines are followed to ensure that any stress associated with anaesthesia is kept to a minimum. The imaging

chamber provides warmth to animals whilst under anaesthesia, and animals are continuously monitored on removal from anaesthesia until recovered.

Blood sampling via the tail vein is not expected to cause adverse effects beyond the transient discomfort of the blood sampling itself, as the sampling volumes are low and the total volumes collected are always within the limit of 15% total blood volume in 28 days recommended by the national centre for the 3Rs (NC3Rs).

Intraperitoneal injections (injections into the abdominal cavity), which may be used for some initial inoculations, dosing of enzyme substrates for imaging, and dosing of cyclophosphamide for immunosuppression, may cause irritation or inflammation depending on the frequency of injections and the substance being administered. Inoculations via the intraperitoneal route are carried out once only. All other intraperitoneal injections are carried out with particle-free solutions, at a neutral pH, and at a frequency which is unlikely to cause longer-term inflammation. As such, these injections are not expected to cause adverse effects beyond those elicited by the parasitic or bacterial disease itself, or cyclophosphamide as described below.

Immunosuppression with cyclophosphamide is often associated with bodyweight loss, reduced appetite, piloerection (hair bristling or standing up on end, typically indicative of either discomfort, pain or stress) and reduced activity. The adverse response to cyclophosphamide is believed to be largely due to effects related to nausea, based on responses in other species. Immunosuppression also carries an increased risk of opportunistic infections, however actual incidence of this causing noticeable effects in experimental animals is extremely rare.

Mice may be genetically modified. These genetic modifications are not generally expected to affect animal welfare. Genetically immunocompromised mice are housed in Individually Ventilated Cages to minimise infection risk.

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 previous licence, we observed that approximately 55% of all animals used experienced a moderate actual severity. The remaining animals are expected to be mild (for experimental/infection protocols, around 43% of all animals used on the previous licence), or sub-threshold (for the GA maintenance protocol, around 2% of all animals used on the previous 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?

Animals need to be used because there are no reliable alternatives currently available to achieve the objectives of this project. Compounds that show promising activity in laboratory tests may still prove to be poor agents in vivo and therefore there is still no alternative to using animals to select promising compounds for further preclinical testing and clinical trials in humans. It is also a legal requirement to demonstrate safety and efficacy in animal models before clinical trials can begin.

While they are not sufficient alone to prove in vivo efficacy, non-animal methods are used prior to animal work being carried out under this project, to develop, improve and select compounds as potential treatments. Cell-based assays are used to find compounds which are active against the parasites, determine how they work, select those compounds which have appropriate characteristics for use in animals (such as how well they will dissolve inside the intestines, and how quickly they are metabolised), and to screen for various forms of toxicity against cells. Cell-based tests are carried out using cells from humans, and from the species of animals which are likely to be used in efficacy and later toxicology testing. Computer modelling is also used to help improve compound design, to predict the required doses needed to be effective in animals, and to predict potential issues with toxicity or “off- target” activity (the term for when a drug affects something else in addition to what it was designed for).

Some of these cell-based assays, as well as work on infected animals, requires a supply of suitable parasites. Parasites are sometimes difficult to obtain in sufficient amounts from in vitro culture and have to be obtained from animals. The nutritional environment in tissue culture is different to that of the animal host and it is important to confirm that parasite adaptation to in vitro conditions has not affected virulence in animals or susceptibility to experimental compounds.

In certain cases, host tissues or cells (specifically macrophages) are required for in vitro parasite culture.

Genetically altered mouse lines may need to be bought in in batches and maintained for a period of time prior to use on experimental protocols.

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

In vitro culture of parasites was considered as a replacement for the use of animals to culture parasites.

Cell-based antiparasitic assays have been considered as an alternative for parasitic efficacy studies.

Why were they not suitable?

Although much basic biochemical and molecular research is carried out using parasites grown in culture containing human or animal serum or using model organisms, it is not always possible to obtain sufficient amounts of parasite material of the appropriate life-cycle stage for these types of investigation. Additionally, the nutritional environment in tissue culture is different to that of the animal host and it is important to confirm that parasite adaptation to in vitro conditions has not affected virulence in animals or susceptibility to experimental substances. Otherwise, the initial tests of drug activity in vitro and the later efficacy studies in animals could both be compromised.

Cell-based antiparasitic assays are not currently able to adequately predict in vivo efficacy in animals and humans due to the complexity of infection in live animals, and so animal models of efficacy are still required. However, these assays are used to provide information for the triaging of compounds for in vivo efficacy.

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?

Careful experimental design is used to determine that the correct number of animals (neither too many, nor too few) is used to obtain biologically significant results. Generally, for efficacy studies, based on previous experience, group size will usually be 6 animals.

Compounds found to be active will be re- tested at serial dilutions to determine the minimal effective dose. This will usually require up to 5 groups, each with 6 animals, based on a statistical method known as the resource equation method that is used to calculate a reliable effective dose. If we used fewer animals or less doses for potential treatments, there is risk of not adequately and robustly defining the minimal dose that works or the amount of substance the pathogens have been exposed to.

This information is used to predict the correct human dose and what the safety margins are.

For the production of parasites or macrophages, the number of animals used will be as few animals as is required to produce the necessary number of parasites or macrophages to support in vitro assays, or other protocols in this project. The numbers have been estimated based on the numbers required during the previous licence period.

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

By using non-invasive imaging, measurements can be collected from the same animals throughout a study, avoiding the need to sacrifice animals to collect tissues for analysis.

This reduces the number of animals required to obtain information about the levels of infection, whilst also allowing for visualisation of infection levels in other tissues and reduces the influence of individual differences between animals (inter-animal variability) on overall results.

Where blood sampling is required, microsampling techniques are used to enable repeat sampling from the same animals. This reduces the number of animals required to obtain the data, while also reducing the impact of inter-animal variability on results.

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

In vitro assays will be used to triage and guide decision making prior to progression to in

vivo studies.

Efficacy studies are informed by pharmacokinetic (PK) studies performed under a separate project licence. PK studies generally use n=3 animals per route per substance using a single dose acute regimen, or a 4-day dosing regimen. This is deemed sufficient to enable valid decisions to be made. This allows for comparison of the information obtained from in vitro assays against the actual in vivo DMPK profile, and ensures treatments are tolerated at the selected dose level, before they are progressed to larger-scale efficacy studies.

In some circumstances we may be able to share parasites produced by other labs, reducing the numbers of animals we require for parasite production.

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 Chagas disease, in vivo bioluminescent imaging protocols are used to assess efficacy against T. cruzi in mice. Immunosuppression is used in this model to accelerate decision making on parasite cure or animal relapse thus reducing total experimental time and potential additional risk of animal suffering. Rag2KO mice are used to produce T. cruzi parasites, as they show very few observable clinical signs when the number of parasites reaches the scientific end-point.

For visceral leishmaniasis, continuous passage of leishmania parasites in tissue culture leads to loss of virulence and passage through an animal is used to restore virulence.

Historically the hamster was used but they developed clinical signs and had to be humanely killed at approximately 6 months following infection. We have refined this by now replacing hamster with RAG2KO mice for parasite production. These mice deliver sufficient parasite load without observed clinical signs and without the need to be humanely killed during their lifetime.

For the bacterial persister model, the methods we are developing uses an in vivo bioluminescent imaging protocol to assess efficacy against S. Typhimurium. This allows for relapse of infection to be seen at an earlier stage, allowing for the scientific endpoint to be reached at a stage when animals are likely to experience fewer adverse clinical signs from the infection.

Genetically altered or mutant mice may be used as the model of infection (e.g. 8HUM mice) when predicted therapeutic drug levels can be obtained that would otherwise not be achievable in wild- type mice. This allows rapid in vivo proof of concept of efficacy of a new substance.

These rodent models of infection closely mimic the course of the human diseases and the

outcome of treatment with the currently available medicines.

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

Mice are the lowest animal that are suitable hosts for the diseases being investigated in this project.

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

The chosen models have a predictable and reproducible outcome.

The use of trypanosome parasites expressing firefly luciferase has allowed bioluminescent imaging protocols to be developed for assessing efficacy against T. cruzi, avoiding need to use death as an endpoint in the mouse model of Chagas disease.

Previous efficacy studies against visceral leishmaniasis generally required the termination of animals to collect organs for the determination of parasite load. Future studies on visceral leishmaniasis will be refined via the development of a bioluminescent strain of L. donovani to allow for non-invasive imaging in a similar method to that used for T. cruzi.

For the bacterial persister model, the methods we are developing use an in vivo bioluminescent imaging protocol to assess efficacy against S. Typhimurium. This allows for relapse of infection to be seen at an earlier stage, allowing for the scientific endpoint to be reached at a stage when animals are likely to experience fewer adverse clinical signs from the infection.

Manganese in normal rodent chow causes gastrointestinal hyperintensities which result in temporally unstable signals, leading to image-ghosting and decreased image resolution.

To avoid this problem, a commercially available rodent manganese-free chow can be used to improve image quality of the gastrointestinal tract. This manganese-free chow, apart from the omitted manganese, which is available in tap water, is a complete diet and readily available. Feeding a manganese free rodent diet has not yet been investigated as an alternative to food restriction on those study types in this licence where this could add value to the quality of the imaging. This will be explored as it offers a more refined approach to food withdrawal.

During the previous licence period, various supportive measures were tested with the aim of reducing the impact of cyclophosphamide immunosuppression on bodyweight, appetite and overall condition, including the provision of additional enrichment, moist diet, high-calorie liquid or gel diet supplements, and/or dietary enrichments known to be appealing to mice, starting from 10 days before the first administration until full recovery from the immunosuppression. Further options to reduce the impact of immunosuppression on animals shall continue to be tested during this licence period.

Options for the use of voluntary consumption of substances in food or treats are being considered for its potential as a more refined method than oral gavage, for example for infection of mice with salmonella bacteria.
The advice of the NACWO/NVS will be sought on matters of animal day to day care and welfare, as required. Animals will be housed in groups where possible and environmental enrichment provided. Animals will be housed and cared for in conditions that meet or exceed the Code of Practice.

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

LASA good practice guidelines for the administration of substances inform the maximum dosing volumes in mice for each administration route chosen.
Limits on blood withdrawal follow the NC3Rs guidance on blood sampling in mice.

Body Condition Scoring: Ullman-Cullen M and Foltz CJ (1999). Body condition scoring: A rapid and accurate method of assessing health status in mice. Lab. Animal Sci., 49, 319-323.

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

Members of the group receive the e-mail newsletter from the NC3Rs (National Centre for Refinement, reduction, and replacement) as well as information from the local animal users' group about best practices regarding the 3Rs. We attend local training and seek out advice from the Named Veterinary Surgeon (NVS) and the Named Animal Care and Welfare Officer (NACWO).

25. In vivo imaging of wild-type/immunocompromised/transgenic animals

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, Diagnosis, Therapy, Contrast agents

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

Our ultimate aim is to develop novel medical imaging probes for non-invasive detection of disease inside animals and humans. This project will allow us to study and refine novel and existing imaging probes and technologies in healthy animals to assess how useful these might be for detecting 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?

Non-invasive medical imaging has become mainstream and one of the many tools used by

clinicians in modern medicine. Whilst its appeal to patients is the non-invasive nature of these modalities, to the clinician it is invaluable in providing information. For example, imaging can assist with: disease detection for both early screening programmes and diagnoses for illness such as cancer, stoke, Alzheimer's disease; treatment planning and refining precision of interventional procedures such as radiotherapy or surgery; monitoring of disease and treatment progression or assessing organ function such as in cardiovascular imaging and foetal imaging.

Fundamental to many of these imaging approaches is the use of a contrast agent. A medical imaging probe/contrast agent is a substance which can generally bind to an internal biological target or occupy a particular space (e.g blood or brain). This event can then be detected from outside the body by using an imaging camera thereby enabling non-invasive visualisation. Such non-invasive imaging will typically generate an image/picture, with the contrast agent helping to differentiating between structures and organs. These details allow doctors to understand if there are anomalies, signs of disease or even the extent of disease severity. Imaging can assist with treatment planning but also with the evaluation of whether a therapy is working or not, thereby allowing more timely management of a disease.

Currently, there are many diseases or disease-stages, which can not be detected using a medical imaging device. This is because the appropriate imaging probes or imaging technologies are yet to be developed. In addition, we are often already working at the detection limit of certain instruments and so more research is needed to develop new devices and methods or possibly improve instruments already in the clinic, to allow us to enhance detection and obtain better image quality.

In this project, in a manner similar to testing new medicines, understanding the behaviour of novel imaging probes in healthy subjects is a fundamental step prior to assessment of their potential and usefulness in disease conditions.

This work will also provide an understanding of how long an imaging probe remains in the body, what it's route of excretion is, how much is removed and how much remains at the target site/organ and for how long. This information becomes particular useful when we are imaging the behaviour of a novel/existing therapeutic.

Establishing baseline data is fundamental to improving and refining an imaging agent, all of which becomes crucial to future evaluations in a disease setting.

Equally, validation is required to demonstrate that the images obtained truly reflect the processes in question.

Within this project, novel areas of study include the application of non-invasive interventions (e.g. low frequency ultrasound-LIFU), which can activate an agent (e.g. microbubbles) which, in turn, can transiently mechanically permeabilise membranes, like the blood brain barrier. This momentary membrane alteration could then allow a therapeutic, which ordinarily can not pass through, to penetrate and reach the diseased organ or tissue. These types of studies would allow us to study directed and localised interventions such as photoactivation, LIFU, photoacoustic applications, to enhance therapy uptake by target tissue or activation of contrast agents/potential therapeutics.

Improving and developing novel imaging instrumentation is an important aspect of this programme of work. This includes, assessing how best to combine various imaging modalities to compensate for their individual limitations but exploiting their complimentary

advantages. In addition, we aim to test novel software (e.g. novel Magnetic Resonance Imaging (MRI pulse sequences) or use of Artificial Intelligence (AI) for image analysis and diagnosis) and hardware solutions (e.g. cameras on the tips of biopsy needles, use of multi-mouse beds for simultaneous imaging) to enhance the capabilities of instruments as well as novel prototypes to advance this field of medical imaging.

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

This programme will develop the following outputs:

- Products: New imaging contrast agents and improved chemistry for their synthesis such that these can be made more efficiently and cheaply.
- Devices: Assessment and development of novel imaging instrumentation and methodology, for future application in many diseases. This includes the use of imaging modalities in synergy to produce the most useful data for eventual use in the clinic.
- Knowledge: Validation and demonstrating that the images generated truly reflect the process in question.
- Novel medical uses: Potential use of existing non-invasive technologies to alter physiology or membrane permeability to allow therapeutics or contrast agents access to regions which are not usually accessible.
- Clinical Trials: Work from this programme will guide and inform on the best candidate selections for testing in early clinical trials using new clinical technologies such as Total Body PET being developed in the UK or higher magnetic field MRI cameras.
- All outputs will eventually to disseminated in scientific peer reviewed publications, conferences and meetings.

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

Short-term:

Less animals used: Repeated imaging provides us with the capability to image an individual animal, assess the contrast agents targeting ability, stability and longevity in vivo. By using this technology or indeed using multiple imaging technologies, we can obtain far more data per animal than would otherwise be possible using traditional scientific experimental designs.

Evaluation for progression towards human clinical trials: The immediate benefits of research under this licence would be to allow us to make an informed decision whether to test a new contrast agent or therapy in humans, or return to cell studies or chemical laboratory for further modification. The same is true for the evaluation/development of novel instrumentation, computational methods and combinational imaging modalities to assess their suitability for progression to human clinical trials/studies.

Medium/Longer term:

Chemistry & manufacturing: By developing and evaluating better contrast agents, the quality of imaging data will be improved (e.g. improving affinity for target) **and give better detectability of smaller amounts of disease. For example, in the field of radiochemistry (for diagnosis and/or treatment),** by making radiosynthesis and purification of agents simpler and more robust will lead to shorter production times. In turn, this will result in wider availability to more hospitals without the need for costly cyclotron, radiochemistry equipment and radiochemical expertise in situ. Thus with more widely available contrast agents, more patients will benefit from the technology available.

Future Therapeutic delivery and monitoring: In addition, imaging also has the potential to non- invasively evaluate therapeutic efficacy in patients, providing rapid feedback on therapeutic or interventional effectiveness. An additional area of study would include advances in imaging techniques which have themselves evolved into non-invasive interventions, e.g. photodynamic therapy, photoacoutistic tomography/imaging or Low Intensity Focused Ultrasound (LIFU). These interventions can be non-invasively applied to 1) activate an internalised agent by altering the properties of the agent itself or 2) cause the activated agent to transiently mechanically impact local tissue. For instance, Low Intensity Focused Ultrasound - LIFU activation of pre-administered microbubbles could transiently permeabilise the blood brain barrier thereby permitting enhanced uptake of a contrast agent for diagnosis or a therapeutic for brain cancer.

Novel instrumentation, computational methods and the combination of imaging modalities will benefit both patients and health services through improved diagnosis and clinical decision making. This has already been demonstrated through development of PET/CT and SPECT/CT which are now in routine practice. Clinical MR/PET and MR/SPECT are in clinical use and will extend application of these principles while reducing radiation doses to patients through avoidance of CT scanning. Novel prototypes such as biopsy needles with embedded cameras/needle tracking abilities could improve current clinical practice thereby enhancing the quality of data collection. The immediate benefit that the experiments will provide is the basis for authorised translation of these techniques into clinical use.

Whether directly by the development of new imaging technologies/contrast agents or indirectly by use of imaging as a tool in basic biomedical research: better quality, wider availability and novel applications of imaging technologies. These will lead to better clinical decision making and enhance quality of life for patients. In addition, these could reduce drug development costs and reduce costs for health services.

The beneficiaries will be patients, health services and pharmaceutical companies.

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

We will closely collaborate with academic, clinical and industrial partners to ensure that the developed imaging probes can be tested in human trials and ultimately be commercialised to ensure widespread clinical use of the developed probes for patient benefit.

Similarly, we will closely collaborate with industrial partners to develop prototype software and hardware that can be subsequently shared with other academic centres for wide spread clinical testing.

We will also disseminate the results of this work at conferences and workshops and publish successful and unsuccessful data, in peer reviewed national and internal scientific journals. In addition, we will organise and participate in workshops where we will share our results, unsuccessful approaches and provide hands on training for internal and external academics interested in this work.

Other means of dissemination will include public engagement events via our outreach programmes, e.g. talks at the Pint of Science initiative or active participation in the summer festival of the Royal Society.

Species and numbers of animals expected to be used.

- Mice: 4500
- Rats: 2500
- Rabbits: 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.

Types of Animals: Mice and rats are the mammalian species of lowest neurological sensitivity that provide the necessary size compatible with the scale of resolution or movement associated with the techniques being studied. Resolution of the whole body imaging techniques is of the order of 0.5 – 1mm. Distribution within smaller animals will be beyond these limits.

Less frequently, experiments will be conducted in normal healthy rabbits prior to further work in appropriate disease models tested under other licences (e.g. Imaging Cardiovascular disease). For example, rabbit is the only available clinically relevant model of atherosclerosis (thickening or hardening of the arteries) and events leading to thrombosis (blot clot within blood vessels), which mimics the human condition.

Currently, this clinical event cannot be reproduced in rodents. Thus rabbits are imaged under this particular licence to provide non-diseased control information. This data is valuable in helping us interpret data derived from the diseased animal model.

Genetically modified strains may also be used as long as they remain healthy throughout their use.

Life stages: We will generally use young adults (8 weeks and above for mice and rats and 12-20 weeks and above for rabbits) for our studies. Animals may be allowed to age within limits, if we are studying particular processes/metabolism which may be altered with ageing (e.g mice and rats may be aged up to 50 weeks for controls to atherosclerosis or up to 40 weeks for rabbits). We use adults as these are the appropriate age controls to the ages of animals which we would use in disease models under different licence authorities.

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

Administration of contrast agent generally under anaesthetic (typically by intra-venous injection; anaesthesia may not always necessary). This could be administered via a variety of routes, ranging from intra-peritoneal to possibly through food/water. Duration is momentary and may be repeated through the study plan.

Typically performed once particularly under non-recovery conditions.

In some cases, **an additional molecular or cellular agent** may be administered, e.g. an inhibitor of the specific process, to modify the biodistribution of the contrast agent. Duration may be momentary or administered via food/water. This generally, may be given once prior to step 1, under terminal anaesthesia or may repeated through the study plan e.g. to augment and sustain target receptor expression over a few days.

Imaging by either PET/CT, SPECT/CT, MRI, Optical, Ultrasound, Photoacoustic technologies or other imaging modality or combinations thereof, will be performed under general anaesthetic. This is mainly to keep the animal completely still during image acquisition, which is critical to obtain the best image quality possible. Typically the duration is once a week for one hour, but this frequency may be increased when combined with different imaging technologies.

These three steps would often be combined at the same time, so the animal would be anaesthetised and unaware of the procedures. We have extensive experience anaesthetising animals with very good recovery outcomes and return to normal behaviours post anaesthesia sessions.

Less frequently, non-invasive interventions may be applied to mechanically alter either the contrast agent itself or transiently increase permeability of a membrane barrier (e.g. blood brain barrier) to permit enhanced uptake of a contrast agent/therapeutic. Examples of this include application of Low Intensity Focussed Ultrasound (LIFU), photodynamic therapy or photoacoustic therapy. Typical duration may be up to 30mins and applied once or up to three times a week.

Less frequently, mice may be housed singly in cages with a grid floor post- Administration of contrast agent or Imaging , to collect radioactive faeces/urine for no longer than 3 consecutive days to obtain more complete information regarding the fate/stability of the radioactive contrast agent (e.g. a long- lived antibody).

In some cases both Administration of contrast agent and imaging may be repeated to obtain time dependent data or to image consistency between imaging sessions ('test re-test').

The final imaging session will be under terminal anaesthetic and is generally followed by tissue removal for further data extractions such as: radioactivity measurement to provide additional quantitative biodistribution and dosimetry data; histology or metabolite data.

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

We do not anticipate adverse events to the animals due to the animals themselves (irrespective of genotype) being in good health throughout.

Generally, administration of contrast agents, novel contrast agents in themselves or use of novel imaging technologies is not expected to cause adverse events.

However, on rare occasions novel compounds may be less well tolerated than expected but animals will undergo greater monitoring and humane endpoints will be triggered as soon as possible, if needed.

Administration of blockers/modifiers or enhancers of target expression/availability or therapeutics may cause some mild adverse effects, such as ruffled coat or some weight loss of no more than 10%. The duration of these adverse events are generally expected to be transient and resolve within 48h.

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 ~ 43%; Mild ~51%: Moderate ~6% Rats: Non recovery ~ 66%; Mild ~26%: Moderate ~8% Rabbits: Non recovery ~ 60%; Mild ~30%: 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?

We require the use of animals because:
Bio-distribution in whole organisms (i.e. tracking the injected agents route/ accumulation and excretion through the body), with intact circulatory systems, biological barriers and excretion mechanisms, is key to clinical use.

To validate the mode of action new/improved molecular imaging agents, experiments are required that cannot be conducted in humans for ethical and scientific reasons.

Data generated from this body of work may be used to inform whether to go forward to human clinical applications. Regulatory agencies require animal data to demonstrate safety and efficacy before molecular imaging agents or therapeutics (that can be validated through imaging) can enter human trials.

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

In some limited cases, absolute replacement using humans is a possibility, e.g. when contrast agents that are already used in humans are evaluated for clinical utility and uptake mechanisms. If this is feasible and allowed by the regulatory agencies we will do so.

Prior to all in vivo work, human and animal cell- and tissue-based methods will be used as relative replacements to answer as many research questions as possible and build solid hypothesis to be subsequently tested in vivo. This includes:
experiments in the laboratory, designed to determine target-binding efficiency, agent toxicity to cells, agent stability in cultured cells/tissues/serum;
'Phantoms' are objects used as substitute or mimic for human tissues to ensure that systems and methods for imaging the human body are operating correctly. So 'Phantom' experiments will be to demonstrate function and capability of the new instruments prior to further testing in live animal settings.

Any new/improved molecular imaging agents or instruments, which are found to be unlikely to succeed in animal or later human trials, will be eliminated at this stage.

All alternatives are limited in the amount and quality of data they can provide.

Why were they not suitable?

Alternatives are not suitable to replace the whole animal as yet, because non-living animal/human alternatives cannot accurately replace the complexities of the interactions of novel probes in whole body systems such as the circulation or immune systems nor intact organs and membranes.

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 preclinical teams within our School have worked for more than 15 years on similar projects. Therefore, based on our extensive experience using healthy animals, we estimate this number based on previous experience and types of projects in the pipeline.

We also have new grants which have been awarded following peer review and we have factored in the types of experiments and animals proposed herein to account for future work 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?

As we have access to our archived imaging data from the past 15 years, it is possible to re- examination/re-evaluation previously acquired imaging data. By doing so, we may be able to avoid starting a project from scratch or running pilot experiments, thereby reducing animal numbers.

Our researchers are able to access online tools such as the NC3Rs Experimental Design Assistant during experimental design but all experimental protocols are also assessed by a number of colleagues (research group leaders, myself/imaging scientists, other collaborative scientists, NTCO, NACWO) from inception to final approval.

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

Management: Our animal unit has implemented a new Mouse Colony Management system which is leading to efficient breeding management of colonies through better and clearer data generation. This assists with oversight, planning and control of animal numbers.

Imaging technology: For example, in traditional scientific design, if we were to explore the in vivo behaviour of a tracer over 6 time points, this would require a group of animals for each time point. Each group of animals would culled at their designed time point to allow analysis of the tracer within tissues and organs. If we imagine that 5 animals were

needed per time point, then the whole study plan would requrie 5 animals x 6 timepoints: 30 animals.

In contrast, 'imaging' to determine tracer distribution would allow **repeated time-dependent measurements on the same animal** as animals are only killed at the last time-point. Thus, in this example study design, we could use only one sixth of the animals, i.e. 5 animals based on the above example. Since each animal serves as its own control/base-line, the data are statistically also more robust, which in turn leads to further reduction as smaller cohort sizes are required (because inter- animal variability no longer needs to be considered at the experimental design stage).

Moreover, not only contrast agent distribution in vivo, but potential time-dependent and unexpected re- distribution can be detected through serial imaging. All these attributes contribute to a greatly improved benefit: cost ratio (benefit=data quality/quantity, cost=animal numbers/procedures).

Pilot Studies: Using pilot studies coupled with imaging, allows us to use far few animals in determining the best contrast agent candidates to progress and to design the optimum definitive experimental study going forward.

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.

Species: Mice and rats are the species of lowest neurophysiological sensitivity that provide the necessary size compatible with the scale of resolution or movement associated with the techniques being studied. Resolution of the whole body imaging techniques is of the order of 0.5 - 1 mm.

Distribution within smaller animals will be beyond these limits.

Less frequently, experiments will be conducted in normal healthy rabbits prior to further work in appropriate disease models tested under other licences (e.g. Imaging Cardiovascular disease). For example, rabbit is the only available clinically relevant model of atherosclerosis (thickening or hardening of the arteries) and events leading to thrombosis (blot clot within blood vessels), which mimics the human condition.

Currently, this clinical event cannot be reproduced in rodents.

Models: All experiments will be conducted in healthy animals irrespective of their genotypes.

Methods: Pilot studies are small experimental groups which help us to decide quickly how best to design a statistically and scientifically valid experiment. Thereby helping develop a

larger higher quality study design and reduce possible suffering.

Generally, inhalation anaesthesia will be used for restraint during the imaging sessions but this also allows for fast recovery post anaesthesia. In addition, we aim to combined steps under one general anesthesia session to minimise distress or transient pain, these may include: blood sampling, contrast agent administration, weighing and imaging techniques.

Moreover, there would be full and complete recovery between periods of anaesthesia and/or food withdrawal; rehydrating of animals during long imaging sessions; monitoring of respiration and/or cardiac function and maintaining body temperature during imaging sessions.

We now have the latest models of our preclinical nuclear medicine imaging cameras (PET/CT and SPECT/CT) and these are able to acquire data over much shorter periods of time, thereby shortening our imaging sessions and as a consequence, the length of anaesthesia the mice or rats are exposed to. Finally, we have also in place, the ability to scan up to 4 mice simultaneously or indeed 5 mice using our Optical imaging systems, which increases the workflow speed which is refinement over previous methodology.

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

Studies in less sentient animals cannot be used to replicate the complexity of blood flow, circulation, interaction of novel contrast agents with intact organs and membranes which could then be extrapolated to humans.

In addition, distribution of novel agents within smaller animals will be beyond the detection limits of both patient scanners as well as the current in vivo preclinical scanners used to image live animals.

However, the majority of the animals undergoing regulated procedures will be performed under non recovery anaesthesia under this programme of work, which minimises distress and reduces the severity experienced by the animal.

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

The majority of experiments will be conducted under non-recovery anaesthesia, which means that, at worst these animals will have only experienced the induction with anaesthesia.

Animals which are recovered post imaging, with or without blockers or physiological modifiers, will experience a Mild severity. Inhalation anaesthesia will be used to restrain animals during imaging, but this step is generally coupled blood sampling or administration of substances to minimise transient pain and distress. In addition, full recovery between periods of anaesthesia will be implemented.

During imaging sessions the following will be conducted: re-hydration during long imaging sessions; respiration/cardiac function monitoring to optimise anaesthesia delivery; monitoring of body temperature monitoring/maintenance. When novel compounds or dosages are being studied, small pilot experiments will help refine the experimental conditions prior to a larger more definitive experiment. Finally, prior to novel substances usage, the veterinarian will be consulted where appropriate, and animals will undergo more frequent monitoring post administration to minimise any welfare impacts.

We routinely use monitoring scoring sheets to objectively record animal health indicators and weights. These help us to pick up any adverse events as early as possible, allowing us to either bring the experimental endpoint forward, obtain veterinary or welfare advice or cull the animal as appropriate.

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

We will follow established published guidelines to ensure experiments are conducted in the most refined way. These includes:

The Responsibility in the use of animals in bioscience research produced by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

The Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986.

The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) are a checklist of recommendations to improve the reporting of research involving animals – maximising the quality and reliability of published research, and enabling others to better scrutinise, evaluate and reproduce it.

Accepted limits of volumes and frequencies when administrating compounds and anaesthesia (LASA Good Practice guidelines).

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

We will stay informed by updates from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) website and seminars on the 3Rs organised within and outside of our institution.

Additionally, we have direct support and contact with NC3R's Programme Managers who supports the application of the 3Rs within our institution. Our institution has an internal 3Rs sub-group and a 3Rs Champion to promote advances in this field to all users. These resources provide expert advice and coordinate the sharing of best practice.

Through our local AWERB, we have access to the latest developments, advancements and information relevant to this project.

26. Investigation into pregnancy-related diseases and maternal and early-life therapeutics to improve maternal and offspring 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

Key words

Cholestasis in pregnancy, Glucose intolerance in pregnancy, Offspring health, Therapeutics, Cold exposure

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?

We will study pregnancy-related changes in bile acid, sugar and fat metabolism, both in health and using models of pregnancy-related diseases. We also aim to test therapies for these diseases to understand how treatment can improve maternal, fetal and offspring health. Lastly, we will study early- life interventions (to be started after birth) to improve the health of offspring born to obese mothers with poor blood sugar control, or in young mice with diet-induced obesity in early 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?

Approximately 15% of pregnant women have a metabolic disorder (changes to the way the body handles nutrients) of pregnancy. One example is intrahepatic cholestasis of pregnancy, the most common liver disorder in pregnancy where bile acids, which help digest fat, accumulate in the blood. When the mother's bile acids are high, this increases the risk of stillbirth and preterm birth (early delivery of the baby). Gestational diabetes mellitus is another pregnancy-related disease where women have increased levels of sugar in the blood that is first seen in pregnancy, and that often resolves after delivery. It can cause babies to be large and may result in birth problems such as obstructed labour and/or medically-induced early labour resulting in premature births. The offspring of pregnancies affected by these two disorders also have higher rates of obesity and related metabolic disorders in later life. This contributes to a vicious cycle of metabolic disease over generations that is contributing to significant global disease burden and public health costs.

Although some things are known about the causes of these diseases and how they affect different organs, there are still many factors that are not understood. There are also very limited treatment options during pregnancy. Treatments that are currently used do not always address all issues for affected women or their children, and not a lot of information is available about their long-term impacts on offspring health.

Therefore, more research is needed to understand the metabolic changes that cause pregnancy-related diseases and to investigate new treatments to improve long-term health.

In addition to therapies in pregnancy, we want to investigate interventions starting in early life in offspring of mothers who had a disease in pregnancy or in young mice that are affected by metabolic diseases. We are interested in cold exposure therapy where offspring are placed intermittently in a cold environment to boost their metabolism.

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

We will generate new data to understand:
The effect of pregnancy-related diseases on metabolism

Whether drug treatments during pregnancy show promise for improving these diseases

The metabolic health of the offspring of affected pregnancies

Whether cold exposure therapy can improve health of offspring from mothers with gestational diseases or of young mice with metabolic diseases.

Our findings will be distributed to the scientific community through publication of academic papers, and, where relevant, to the general public through charities that specialise in pregnancy-related diseases via the charities' websites and/or social media.

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

The data generated from this work will have short-term benefit to other researchers who are also studying pregnancy-related diseases. These researchers will be able to build upon any findings as to how changes in metabolism occur during pregnancy and are made worse in maternal metabolic diseases.

Our work testing new medicines to improve the outcomes of diseases of pregnancy will

benefit pharmaceutical companies that invest in strategies to tackle these diseases. This research is studying new ways that these diseases may develop and will provide insights into areas that could be targeted by drugs or other treatments.

In the longer term, this work may also impact clinical care, as drugs/interventions tested in this project may then be taken forward to clinical trials to improve patient outcomes.

Our work will also include cold exposure therapy, either to improve health in offspring from mothers with pregnancy-related diseases or to improve metabolic dysfunction in young obese mice. This could be used as a non-drug treatment in a medical setting in the medium to long term if the results are positive.

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

Our findings will be disseminated through publication in peer-reviewed academic journals and presented at national and international conferences. We will also communicate our findings via social media. Where relevant, we will work with charities which specialise in pregnancy-related diseases to ensure the results of our work are publicised in a helpful and educational manner. This work will assist them in giving constructive advice to women with intrahepatic cholestasis or gestational diabetes mellitus.

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.

This work cannot be performed in humans as it is unethical to manipulate human pregnancy or obtain the tissues and other samples required to investigate ways the disease develops in detail. Instead, we are using mice for our work, as they have been well-characterised with extensive publications from ourselves and others and as such, provide the best models available. Adult mice show similar control of blood sugar to humans and share many of the same changes in pregnancy (e.g. similar reproductive hormone profiles, increased bile acids in the blood, increased fat tissue and altered sugar regulation), making them especially suitable for pregnancy experiments. In addition, human intervention and offspring follow-up studies are time-consuming, while the relatively quick life cycle of rodents allows us to study the consequences of maternal disease on adult offspring within a meaningful timeframe.

This makes it possible to assess offspring health in young adulthood as well as in older age. Lastly, cold exposure can stimulate certain types of fat to burn energy as heat. This type of fat is present at the highest level in newborn and young mice.

Therefore, these life stages are most suitable to test the effects of cold exposure therapy on metabolic health, as early life is when this potential therapy would take place in the human clinical setting.

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

There are a number of different treatments we want to explore. The typical procedures carried out on a mouse for each objective are shown in a diagram at the end of this section.

Objective 1: Investigate metabolic influences on susceptibility to poor bile acid and/or poor sugar control in pregnancy.

We will breed mice which have been genetically modified to alter genes involved in the diseases or treatment targets we are interested in. We will take a piece of tissue from the ear from these mice to determine its genetic makeup (e.g. whether they have the genetic modification of interest or not).

Objective 2: Investigate the impact of liver disease in pregnancy and treatment on maternal and offspring health

These investigations will use two different models. Both models will require female mice to be mated with a male mouse. Female mice will be singly housed for the duration of the pregnancy.

For the diet model (i) mice are fed an altered diet or a nutrient-matched control diet. Treatment may be given in the water or directly into the mouth. If treatment is given directly by mouth, this will be done once daily during the course of pregnancy or from partway through pregnancy.

For the drug-inducing disease model (ii) mice will be placed on a standard diet or a diet containing medicine in the feed or in their water. From late pregnancy up to birth, mice will receive a daily injection under the skin to induce the disease.

Some mice from both of these models may be given drugs either from the beginning of pregnancy or starting during pregnancy to see whether these medicines can help manage the impact of the disease. Some mice will have their blood sampled up to 2 times during pregnancy. Some mice on the diet model (i) may be placed in a special cage for up to 24 hours to monitor water and food intake and to collect urine and faeces. This may be done once prior to pregnancy to help them get used to the cage, and once during pregnancy. Some mice from both models may be humanely killed during pregnancy. Others will be allowed to give birth so that we can study the offspring health. If the mother gives birth, pups may be humanely killed before weaning or some litters may be reduced to a standardised number a few days after birth. Pups from mothers who underwent either of these models may be weaned and long-term offspring health investigated. The mother will be humanely killed at this point.

Offspring may be given a high-fat/-sugar or nutrient-matched diet for up to 9 months.

These mice may undergo glucose or insulin tolerance tests to measure their sugar control up to 6 times. The tests will involve food restriction for up to 12 hours, an injection of a harmless substance and pricking of the tail to measure blood sugar.

Blood samples may be taken up to 36 times over 12 months. Some mice may be placed in a special cage for up to 72 hours (maximum 6 times over 12 months), and some mice may have body composition scans (up to 12 times) lasting around 10 minutes which happens when the mouse is awake and restrained. All offspring will be humanely killed at the end of the experiment (maximum age 12 months).

Objective 3: Investigate the impact of treatments on maternal and offspring health in models of maternal obesity and/or diabetes in pregnancy.

Female mice may undergo a diet change up to 12 weeks prior to mating to induce obesity and/or sugar intolerance. Mice may be provided with medicine for up to one week before mating and throughout pregnancy; these may be given by mouth (most likely in the diet or drinking water). Female mice will be singly housed for the duration of the pregnancy and may be singly housed for up to 1 week before mating.

Adult female mice may have their blood sampled up to 4 times at intervals before, during and after pregnancy. Female mice may also have their sugar or insulin control measured, with only one test taking place in pregnancy. The tests will involve food restriction for up to 12 hours (6 hours in pregnancy), an injection of a harmless substance and pricking of the tail to measure blood sugar. Some mice may also have non-invasive blood pressure measurements and imaging techniques performed (imaging performed under general anaesthesia when necessary).

When sugar/insulin tolerance testing or imaging under anaesthesia are performed during pregnancy, the pregnant female and her fetuses will be humanely killed during pregnancy no more than 48 hours after the procedure to avoid any long-term harm for the offspring.

Pregnant female mice and their fetuses may either be humanely killed during pregnancy to examine the effects of the disease and/or treatment on the mother and the fetuses, or the pregnant female is allowed to give birth, generating offspring.

Offspring may be followed into adulthood to determine any long-term health outcomes associated with maternal metabolic disease and/or maternal treatments.

Mice may be singly housed where it is not possible to keep them with another mouse. Offspring may be given a high-fat/-sugar or nutrient- matched diet for up to 9 months. These mice may undergo glucose or insulin tolerance tests to measure their sugar control up to 6 times. Blood samples may be taken up to 36 times over 12 months. Some mice may be placed in a metabolic cage for up to 72 hours (maximum 6 times over 12 months). A metabolic cage is a special type of cage where the mice have reduced bedding and enrichment. They have free access to food and water and the cage will collect their urine and faeces and will measure changes in the air composition to determine how their energy needs are changing.

Some mice may have body composition scans (up to 12 times) lasting around 10 minutes which happens when the mouse is awake and restrained. Some mice may have non-invasive blood pressure measurements and/or ultrasound imaging performed under short-term general anaesthesia of maximum 1 hour when necessary, at three occasions during their lifetime.

A small proportion of offspring may have a sensor implanted to study function of the heart and large blood vessels. These mice will be placed under anaesthesia and allowed to recover before measurement of blood pressure and heart rate in an awake and undisturbed animal. Other offspring may have tubes surgically inserted to give sugar and insulin, with the purpose of measuring blood sugar control. Only one of these two surgeries will be performed in the same animal. All offspring will be humanely killed at the end of the experiment (maximum age 12 months).

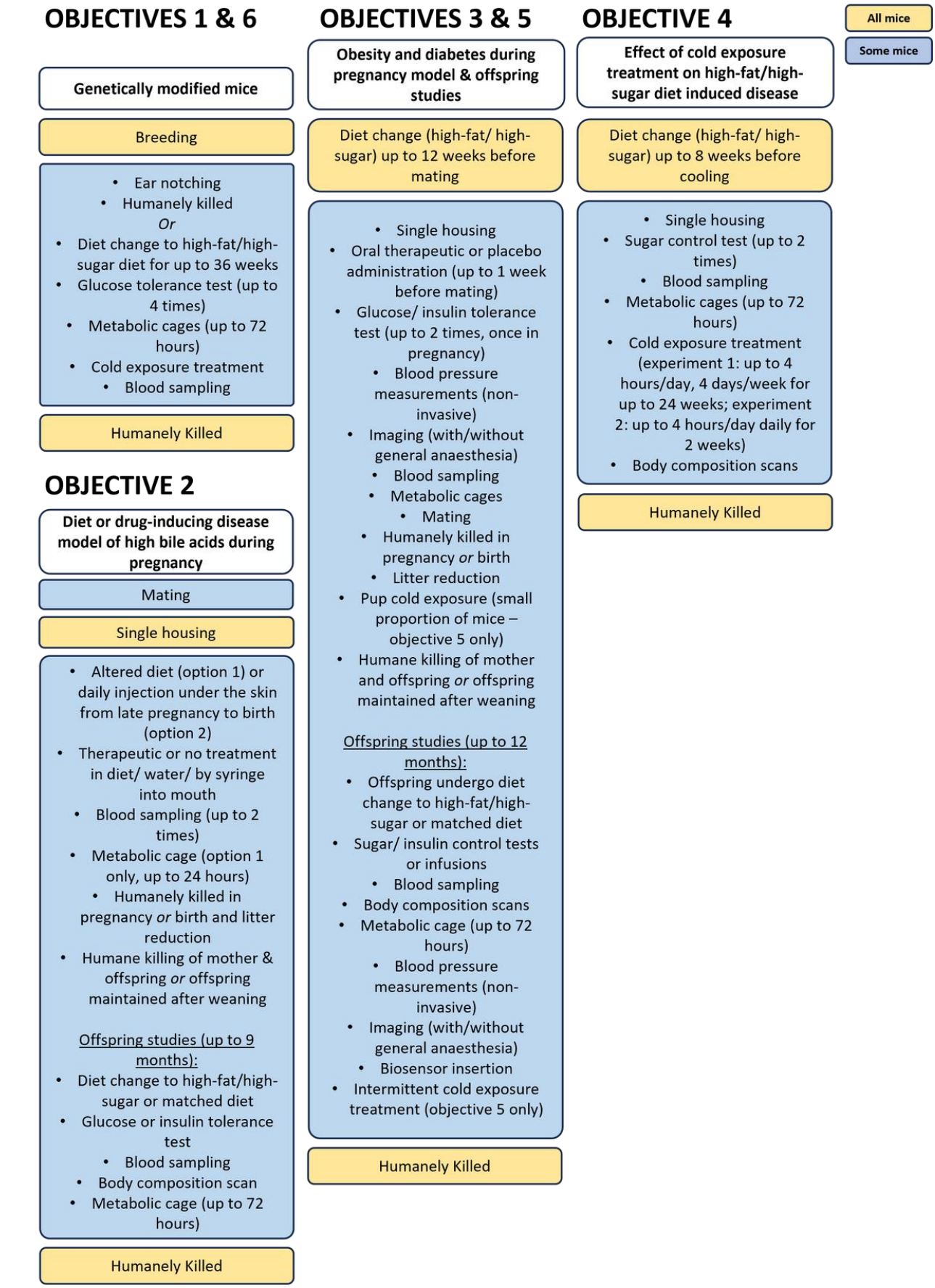
Objective 4: Investigate cold exposure therapy to improve early-life obesity
Young mice will be placed on a high-fat/high-sugar diet for up to 8 weeks prior to cooling therapy starting. Mice may be singly housed where it is not possible to keep them with another mouse. During this time, mice may undergo one sugar tolerance test and one insulin tolerance test which will be at least 1 week apart. Mice may also be placed in a metabolic cage for up to 72 hours (maximum 6 times over 36 weeks). Mice will be placed into a temperature-controlled chamber (down to 4°C) or maintained at room temperature with free access to water. This will occur for up to 4 hours/ day and will be cooled for up to 4 times/ week for up to 24 weeks (option 1), or daily for up to 2 weeks (option 2). Mice may have blood sampled before and after the cooling once per week where mice are cooled over a duration of up to 24 weeks (option 1), or three times over 2 weeks (option 2). Nearing the end of the cooling therapy period, mice may undergo another sugar and /or insulin tolerance test to determine whether cooling therapy has improved blood sugar control. Mice will be humanely killed at the end of the experiment (maximum 36 weeks).

Objective 5: Investigate cold exposure therapy for offspring from obese mothers with poor glucose control in pregnancy
Pre-weaning mice may be cooled up to 6 times during the pre-weaning period and after weaning may be cooled again intermittently. Pups will be placed alone in a dry container floating in a water bath set to a cold temperature (down to a minimum of 15 degrees) or a temperature comparable to what they would experience in the nest with their mother for up to 3 hours at a time without bedding.

After weaning, mice may be placed on a high-fat/high-sugar diet or nutrient-matched control diet for up to 8 weeks before the cooling therapy protocol is started and will continue with the same diet throughout the duration of the cooling therapy experiment. Mice may be singly housed where it is not possible to keep them with another mouse. Mice may undergo cold therapy which will involve ntermittent periods in a cold chamber (from 3 times/ week to daily depending on the experiment, for up to 4 hours/ day). This will occur over a period of 1 week to 9 months. Mice may undergo a sugar and/or insulin tolerance test prior to diet change and may undergo a further two sugar and/or insulin tolerance tests over 9 months. Mice may be placed into a metabolic cage for up to 72 hours, for a maximum of 6 times over 9 months.

Some mice may also have up to two blood samples taken on weeks where no sugar/insulin tolerance tests are performed up to a maximum of 36 times. Mice will be humanely killed at the end of the experiment (maximum 12 months).

Objective 6: Understand mechanisms of action for activation of brown fat in lean and obese mice
Genetically altered or normal mice may be fed a high-fat/high-sugar diet for up to 36 weeks. Mice may be singly housed where it is not possible to keep them with another mouse. During this period, mice may undergo cold exposure (up to 6 hours/ day for 3 days/ week for up to 24 weeks). Mice may have up to a total of 4 sugar or insulin control tests and may have multiple blood samples taken. Mice may have blood sampled before and after the cooling once per week in the weeks where sugar/ insulin control tests aren’t performed. Mice may also be placed in metabolic cages for up to 72 hours for a maximum of 4 times. All mice will be humanely killed at the end of the experiment (maximum 9 months).



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

Single housing in pregnancy
Mice are social animals and therefore the experience of being housed on their own may be stressful, particularly for female mice and particularly in pregnancy.
Impact of high-fat/high-sugar dietary modification

Animals are expected to feel mild to moderate discomfort due to dietary changes associated with weight gain. Some animals will be given a diet to cause obesity and may experience adverse effects secondary to their obesity, such as having an oily fur coat, increased drinking and urination, or overgrooming which can lead to sore development. Prolonged high-fat diet can also cause overgrown teeth due to the soft diet.

Impact of cholic acid dietary modification
Adding a bile acid to the diet will cause high bile acid levels in the blood and may cause some discomfort in the mice. In our experience, mice do not show signs of being unwell on the cholic acid diet.

Impact of injections under the skin to cause cholestasis in pregnancy
Daily injections during late pregnancy to cause cholestasis are likely to cause moderate discomfort, including bruising and rarely also sore development, alongside stress. The drug given is likely to cause discomfort and have adverse effects on the liver. This drug is likely to cause preterm labour and stillbirth of some pups.
Impact of oral dosing with therapeutic during pregnancy

Providing medications in the food or drinking water during pregnancy is unlikely to have adverse effects. However, mice may experience a mild degree of stress during pregnancy if treatments are directly administered orally by an investigator, as this requires short-term restraint of the animal.

Impact of sugar/insulin tolerance tests and blood sampling
Only brief discomfort is expected to result from injections and blood collection, which should pass within minutes. Food restriction required for certain procedures is likely to produce a modest degree of weight loss which is typically regained within 48 hours. Mice will always have access to water. There is a small risk for the mice to have low blood sugar following food restriction or an injection with insulin.

Impact of metabolic cages
Mice that are placed in metabolic cages may experience mild stress, cold and discomfort (especially during pregnancy) because these cages will have minimal bedding required to determine food/water intake, urine and faeces production, and changes in energy n.

Surgical Procedures
Animals that have biosensors implanted or tubes inserted are likely to experience moderate discomfort from the surgery for a few days, which will be managed with pain relief medication. There is also a very small risk of developing a post-operative infection or wounds reopening which will be treated following advice from the Named Veterinary Surgeon. There are risks associated with short-term anaesthesia including a temporary mild decrease in heart rate, drying of the eyes and a small risk of not waking up from the anaesthesia.

Impact of cold exposure
Mice undergoing cold therapy are expected to feel mild discomfort during the procedure that will resolve shortly after returning to room temperature. A small amount of stress is

expected with procedures that involve handling which will be minimised as much as possible through adequate training of the investigator. Mice lose weight during the cooling procedure; however, they typically regain the weight lost within 24-48 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)?

Mice: 5% sub-threshold, 25% mild, 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?

Pregnancy is a very complex process, and trying to understand signalling changes in uncomplicated pregnancies and disease cannot be fully replicated using cell culture or computer modelling methods. It is not ethically or practically possible to experiment in pregnancy or obtain samples from the tissues of interest for this research from pregnant women and their children, therefore it is necessary to use mouse models. Similarly for the cooling studies, we are interested in multiple organ responses, and the signalling between these organs which might result in improved health which cannot be explored without investigating the whole body system.

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

We considered using standard cell culture as well as organoid culture (a mini organ system) of the organs of interest (e.g. liver). Where possible, for investigating how new therapies work we will use cell/organoid culture and to look for blood markers of disease we will investigate stored banks of human samples where feasible.

Why were they not suitable?

We are interested in cross-organ communication and changes in different maternal and fetal organs during pregnancy, or how organs respond to cold challenge: both of these aims involve whole body signalling. Therefore we cannot use alternative approaches to animal models, as cell or organoid culture would not encompass all these changes or show us how different organs signal to one another. Additionally, we are investigating long-term health consequences for offspring of mothers with a pregnancy-related disease and cannot do this with a cell/organ 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 estimated the number of mice based on the predicted numbers of experiments, the numbers of experimental groups and the number of mice in each group. All estimates are based on similar studies we have conducted in the past using comparable protocols or based on peer-reviewed studies.

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

The proposed experimental designs and methods of analysis were discussed with a statistician so that we can maximise the information obtained from the minimum resource. This was done alongside using the NC3Rs Experimental Design Assistant.

We will use in vitro work using primary cells or cell lines to determine the action of potential treatments which will inform which treatments are most effective; only these will then be tested 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?

Where possible, we will use the same animal to address multiple research questions (e.g. the effect of diabetes on fat tissue and on liver tissue) and will collect as many relevant tissues as possible from these mice to be stored so that if future questions arise, the experiment does not need to be repeated. Where it is feasible, samples or data from mice that do not have disease induced or undergo treatment will be used as controls for multiple experiments where these experiments can be conducted at the same time. No additional procedures will be applied to these 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.

We are using mice to investigate the aims of our project.

Handling and housing
Mice will be handled to acclimatise them to being picked up for procedures. This will reduce stress during the procedures. Unless animals are required to be singly housed for experiments that cannot be performed otherwise, mice will be kept in pairs. For

experiments that require diet/water consumption monitoring, this will be done through short periods (<72 hours) in metabolic cages where feasible rather than weighing diet/water which would require single housing for a longer period.

Oral dosing for gestational diabetes mellitus
The treatments studied for use in a mouse model of diabetes in pregnancy are oral treatments, therefore they are less invasive than the current gold standard treatment of insulin injections. These treatments are safe and commonly given in human pregnancy. They have also been used in previous mouse pregnancy studies performed by members of the group, where they were not associated with poor health in the mother(e.g. loss of appetite, pregnancy loss, effects on mating). The treatments under investigation for this disease will only be given to female mice who have glucose intolerance (inability to regulate their blood sugars) from feeding a diet high in fats and/or sugars, and not to control mice (as this is not done in clinical practice either). This is in line with the ARRIVE guidelines (NC3Rs, guidelines for performing more humane animal research) so that the minimal number of animals needed is used. Medications will be provided in the diet or drinking water where possible to minimise stress and discomfort to the animal. Direct oral administration of a medication by an investigator will only take place if treatments cannot be given in the diet or drinking water or if an exact daily dose is required to be given. In this case mice will first undergo the technique before pregnancy to minimise stress associated with a new treatment method during pregnancy.

Subcutaneous (under the skin) injections of vehicle or cholestasis-inducing drug
Injections will be given daily for pregnant mice in this protocol from day 16 of pregnancy onwards. This method of inducing cholestasis is required to cause high bile acids to be produced internally (rather than in the diet) which is needed when investigating whether we can block re-absorption of bile acids from the gut after a meal.

Glucose/insulin tolerance tests and blood sampling
Blood samples will need be collected to measure blood glucose, hormones and other molecules of interest. This will be done via tail bleeding, which is less invasive than collecting blood from other sites in the body. The number of blood samples that can be taken is limited per animal and the procedure will cause no more than temporary pain. This procedure is a less invasive alternative to procedures like continuous glucose monitoring, which requires a probe to be surgically inserted into the animal's blood vessels and may only be performed if the research question justifies it. A small proportion of mice may undergo the more invasive gold standard method of determining insulin sensitivity that involves surgical implantation of tubes to give glucose or insulin. This technique will only be performed by a trained investigator on the smallest number of animals in instances where the research question justifies its use.

Metabolic cages
Time in metabolic cages will be limited to 24-72 hours to minimise stress in juvenile and adult mice and will be limited to a maximum of 24 hours for pregnant mice. Use of metabolic cages will replace more invasive techniques like prolonged single housing and manual urine collection for food intake and excretion analysis. Metabolic cages will allow us to gain information on energy expenditure as well as clean separated collection of urine and faeces with liquid-repellent sand would not be able to do. Use of metabolic cages in pregnant mice will only be performed once during pregnancy where required, and these mice will be acclimatised to the metabolic cage prior to mating to reduce stress during pregnancy.

Non-invasive imaging

In order to determine the body composition (e.g. fat percentage) of an animal using a technique called nuclear magnetic resonance, conscious mice are placed in a medium-sized tube (allowing movement) for a short time only (approx. 5 minutes). As no restraint or anaesthesia is required, this is a non- invasive and harmless method of determining fat mass that can be used both in pregnant and in non- pregnant mice. Moreover, this imaging technique provides the opportunity to measure body composition (fat and muscle) of an animal multiple times. Therefore, fewer mice will be required to study the development of obesity across the life course.

Mice may also undergo ultrasound measurements to determine heart function or blood flow in key blood vessels (e.g. aorta, placental blood vessels). This technique requires only short-term anaesthesia and is therefore safer than MRI scanning, which takes longer.

Blood pressure measurements
Blood pressure will be measured to determine the effect of maternal metabolic disorders in pregnancy and/or any treatments on the heart and circulation in both pregnant mice and their offspring. This may be done using a non-invasive method involving balloon cuffs that temporarily restrict blood flow to the mouse's tail, much like how blood pressure is measured in humans. As this technique is non-invasive this may be performed in pregnancy or may be repeated at various points in life to measure the development of blood pressure changes throughout the life course. With training the potential stresses of this technique on the animals will be minimised and this procedure is of mild severity.

A small proportion of mice may undergo the more invasive gold standard method of determining blood pressure that involves surgical implantation of a biosensor that can measure blood pressure and heart rate in recovered conscious animals.

Suffering is minimised by using skilled scientists for the surgeries, by working cleanly to avoid infection, and by providing anaesthesia and painkillers during the procedure, by providing painkillers after the surgery, and monitoring of the mice until they are recovered.

Cooling of mice
Where we need to measure temperature in pups, this will be done using a thermal imaging camera rather than a rectal probe, which causes significant damage in pups and would necessitate humanely killed; use of a thermal imaging camera means that no harm is caused to the pups and therefore fewer animals are required to achieve the experimental results.

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

As we are studying pregnancy, we need to investigate mammals as many relevant features of metabolism and reproduction are similar to human pregnancy. Species that are less sentient do not have such similar metabolic and reproductive systems and therefore we cannot use these for our research. We cannot investigate terminally anaesthetised animals as we want to study how the body reacts to disease over the course of pregnancy, and how this affects the offspring, or how early-life interventions can impact obesity.

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

We will constantly review our protocols to refine them to reduce harm to the animal where possible. Animals that undergo harmful procedures will have increased monitoring to ensure their welfare is maintained and will be humanely culled if their suffering becomes

too severe. Mice will be acclimatised before procedures that require significant handling (e.g. repeated injections) so that they are used to being handled. Where stress is likely to be induced such as bedding removal to allow for camera monitoring for pregnancy-related outcomes, environmental changes will be performed gradually over a number of days where possible. When metabolic cages are required during pregnancy, mice will be placed in the cage prior to mating so that they have an experience of the cage pre-pregnancy and thus the environment is familiar and stress is reduced.

Injections will be done by highly trained staff. Where there is a welfare concern for the mouse model of cholestasis using under-the-skin injections, mice will be monitored carefully through a combination of cameras attached to the outside of the cage and by manually checking every 4-10 hours. After daily injections have started, food consumption will be monitored through daily weighing of food hopper from the start of the daily injections, and mouse weight monitored through daily weighing until onset of labour to ensure they are eating. Once labour has started weight changes will no longer be used to monitor mouse health, as often mice do not eat or eat less during labour.

Where procedures have caused swelling e.g. due to injections or surgery, advice will be sought from the NVS for treatment. After operations, mice will be more frequently monitored to ensure recovery, and will be given pain relief both during and after surgery. If mice develop sores from overgrooming or diet-related issues, they will be monitored carefully, and topical treatment given where advised from the NVS/NACWO.

Where birth and pup health details are required , pups will be monitored carefully after birth using cameras where required. This will reduce the number of times the mother has to be disturbed around the birthing period and therefore minimises the stress and risk of miscarriage and cannibalism.

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

We will follow the NC3Rs resource library to ensure that our experimental design is as refined as possible prior to commencing any experiment and will follow the ARRIVE guidelines for reporting our work. We will follow Morton et al. (2001) for guidelines on dosing limits.

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

We will regularly check the NC3Rs website to make sure we stay up to date about any advances, as well as keeping informed about any new techniques that might reduce the need for animal experimentation by reading scientific publications and speaking with colleagues within our university and in the broader scientific field. We will additionally stay up-to-date with in-house resources through the 3Rs advisory group based in our facility, When any advances are made, we will review our protocols and experimental designs to determine whether we can apply this to reduce, refine or replace our use of animals.

27. Provision of Biological Materials

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

Blood, Tissues, Biological Materials

Animal types	Life stages
Rabbits	adult, juvenile
Chicken	adult
Mice	adult, juvenile
Rats	juvenile, adult
Animal types	Life stages
Hamsters (Syrian) (Mesocricetus auratus)	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 provide blood and other animal tissues for subsequent in vivo and ex vivo 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 a continuing need for biological material from known health status animals to support biomedical research projects and to meet regulatory and non-regulatory requirements. Part of this need includes the provision of high-quality animal tissues of the type produced under this project licence. These tissues include blood products (whole blood, blood cells, plasma and serum) and various organs (e.g. lungs, brains and kidneys) for use in a number of ex vivo/in vitro investigations to support both regulatory drug submissions and efficacy work in the human pharmaceutical and veterinary health markets.

Governmental approval is required before a new chemical or substance may enter clinical human or veterinary trials, food products, the workplace or the environment. Further approval may be needed before it can be marketed. There is an expectation, both governmental and public, that these materials should be safe for use, or that their potential hazards are well understood and documented. Guidelines issued by government appointed regulatory agencies specify the types of animal and non-animal studies that must be completed in order to apply for clinical trial authorisation or product marketing approval.

Tissue samples are required to support or develop, validate or calibrate assays for bio-analytes to be measured on supporting or regulatory studies or for ex vivo investigations (for example cell-based assays) supporting such studies.

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

This programme of work will provide quality blood products (plasma/serum/blood), organs and tissues in both fresh and frozen forms to support a wide range of ex vivo and in vitro assays used to support both Regulatory and non-regulatory drug discovery programmes.

The blood products, organs and tissues provided under the authority of this licence may be used in ex vivo and in vitro assays performed to support the development of new drugs, biologics or vaccines for both the human and animal health industry.

These products, if developed, may have an improved efficacy/side effect profile, or novel mechanism of action compared to existing marketed therapies, and therefore have the potential to improve the quality of life for patients with disorders of an unmet clinical need such as, for example, cancer, cardiovascular and respiratory disease, which are amongst the diseases with the highest morbidity and mortality rates.

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

The blood products, organs and tissues provided under the authority of this licence may be used in ex vivo and in vitro assays performed to support the development of new drugs, biologics or vaccines for both the human and animal health industry. These products, if developed, may have an improved efficacy/side effect profile, or novel mechanism of action compared to existing marketed therapies, and therefore have the potential to improve the quality of life for patients with disorders of an unmet clinical need such as, for example, cancer, cardiovascular and respiratory disease, which are amongst the diseases with the highest morbidity and mortality rates.

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

The data from the assays performed will be used in regulatory submissions to the appropriate regulatory authorities or used to help form a picture of the potential of putative

new drugs to be more efficacious, with a better side effect profile than existing therapies, in a wide variety of human and animal health indications. This data may not always be positive, and hence, some of these tests may prevent the further development of such entities, and hence prevent the un-necessary use of animals in efficacy and regulatory testing prior to testing in human or animal clinical trials.

Species and numbers of animals expected to be used.

- Other birds: No answer provided
- Mice: 160000
- Rats: 60000
- Rabbits: 6250
- Hamsters (Syrian) (Mesocricetus auratus): 1250

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 blood and tissues from rodents, birds and rabbits are used extensively to develop new medicines and study biological responses ahead of any subsequent in-vivo animal studies relevant to humans and other animals.

This programme of work will provide quality blood products (plasma/serum/blood), organs and tissues in both fresh and frozen forms to support a wide range of ex vivo and in vitro assays used to support both Regulatory and non-regulatory drug discovery programmes

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

To enable the supply of fresh blood products (Whole blood and blood cells) and frozen plasma, serum and complement from donor rabbits, chickens and turkeys, blood samples will be taken by venepuncture. If large sample volumes are required a temporary cannula may be used for the duration of the sampling. A peripheral vein is used in all species e.g the jugular vein for chickens and turkeys and the marginal ear vein or auricular artery for rabbits.

The typical experience of animals undergoing a non-recovery protocol for the collection of blood 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;

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

For animals undergoing blood sampling and/or tissue collection under terminal anaesthetic, the deep surgical anaesthetic plane will prevent animals feeling any pain.

When animals are blood sampled in a conscious state the 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 subside within typically 3 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)?

The expected severity of this work is non recovery for 100% of animals blood sampled under terminal anaesthetic.

The expected severity experienced by animals undergoing blood sampling while conscious will be Mild. This will apply to 100% of animals on such protocols.

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?

There is currently a requirement for animal products in regulatory testing and this cannot currently be replaced by products produced by ex-vivo methods. There is no current method of producing the blood products and tissues without the use of animals of the type described under this licence

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

Through our local animal Welfare and Ethical Review Body (AWERB) and Named Information Officer (NIO) we will be kept up to date with suitable non animal alternatives.

Why were they not suitable?

At present there are no suitable alternatives for the use of whole blood and blood 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?

Our ability to take several products from each animal ensures that whenever possible animals used for biological materials will be fully utilised and overall numbers used in the wider scientific community can be reduced. We do this by collecting all the blood available per animal when performing a terminal bleed and where demand exists also harvesting tissues. For example, the blood cells from two chickens are routinely shared across six end users on a weekly basis supporting a variety of end goals.

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 used in this licence include building in the ability to re- use animals that have received minimal mild and transient interventions e.g. removal of blood via venipuncture.

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

By using chickens over 1.5kg under this licence we are able to reduce the total number of birds needed to produce the quantity of blood cells required for the regulatory testing, for example in the haemadsorption test pertaining to screening of biological samples, which are then used in the production of biopharmaceutical products.

Compared to the previous licences we have been able to forecast a reduced number of mice and rats needed for use under this licence, with no decrease in the volume of product produced. This is due to collecting the blood products under terminal anaesthesia (isoflurane) as this has increased the quantity collected, as well as led to improved overall quality of product reducing wastage.

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, Rats, Rabbits, Hamsters, Chickens and Turkeys will be used to supply blood and other tissues for subsequent in-vitro or ex-vivo studies.

For the supply of large volumes of rodent blood the method that will be used is recognised as causing the least pain, suffering, distress or harm to the animal is where the animal is terminally anaesthetised to a level of deep surgical anaesthesia.

Once the animal is deeply unconscious, blood is removed directly from the heart by use of a needle and syringe. In addition, where fresh tissue is required, this is surgically removed while the animal remains deeply unconscious. The animal is maintained under anaesthesia for the duration of the protocol and never regains consciousness. Once the

blood and/or tissue is obtained, the animal is then humanely killed by a Schedule 1 technique and death confirmed at the end of the process.

All licensees and technicians are fully trained in regulated procedures and husbandry of experimental animals. The majority of animals used will have procedures performed under terminal anaesthesia so the animals will experience no pain or discomfort other than that caused by the initial administration of the anaesthetic . For donor blood sampling superficial easily accessible veins are used to minimise the length of the procedure and reduce the risk of bruising or haematoma.

Rabbits are towel wrapped for sampling, this reduces stress for the animal and minimises the time taken for the procedure. On occasion local anaesthetic cream (Emla) may be used on a site prior to sampling.

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

Adult animals are used for blood sampling as their larger body size correlates to a larger blood volume, therefore fewer animals are required to obtain the required sample size. Where large blood samples are required from rodents, the procedure will be conducted under terminal anaesthesia.

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

For repeated blood sampling animals will be handled with appropriate care to reduce stress, local anaesthesia cream may be applied before insertion of a needle to remove blood. Mice will be handled using non adversive handling methods such as tubes or cupping.

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

We will follow LASA and NC3Rs guidance on blood sampling.

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

The use of the NC3Rs online resources provides excellent up to date methods for blood sampling techniques in a range of species. The NIO and local AWERB will provide input to this project to ensure best practice occurs.

28. Understanding the effects of stress on brain development and behaviour across the life course

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 development, Stress, Psychiatric disorders, Behaviour, Molecular regulators

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 project aim is to identify molecular mechanisms underlying stress regulation in the brain, linked with adverse behavioural outcomes such as psychiatric 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?

This work is critical, because there is an urgent need to better understand how stress exposure affects the brain, to treat or prevent development of stress-related disease.

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

This project will provide new knowledge regarding how stress ultimately alters brain function, including psychiatric disorders. New knowledge outputs include: Development of more refined animal models, using zebrafish, to understand stress

regulation in the brain and disease susceptibility.

Comprehensive identification of brain regions and cell types altered by stress exposure Identification of the effects of stress exposure across the life course, including effects on neurogenesis, brain aging, and behaviour Characterisation of the effects of genetic susceptibility to stress exposure Investigation of potential therapeutics to ameliorate the effects of stress.

The results will be published in high-impact open access journals with broad readership and presented at national and international conferences. Data from this research will be publicly available (datasets deposited in public repositories and code shared on GitHub), and important results will be shared on LinkedIn, Twitter and ResearchGate. The work will be shared at public outreach activities such as ‘Pint of Science’, ‘The Festival of Science and Curiosity’ and ‘Soapbox Science’.

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

Academic communities: This work is relevant to researchers with an interest in understanding how stress shapes the brain across the life course, since it will identify potential mechanisms through which stress leads to functional alterations in later life.

This work will also be of interest to those studying aging as it will characterise how stress regulation in the brain changes in aging, ultimately increasing disease risk in later life. The new stress and disease models developed during this project will be a valuable tool for the wider stress research community, and these tools will be shared to facilitate advancement of scientific understanding in the field. By publishing our results in high-impact journals with broad readership, and presenting at national and international conferences, the findings will be shared with academics working in other systems, with whom we will build collaborations. This will facilitate more detailed mechanistic studies of potential therapeutics in other models and ultimately humans.

Industry: The molecular targets identified in this project will provide potential targets for the development of drugs to treat stress-related disorders. Validated targets could be ultimately developed into therapies with involvement of pharmaceutical and biotech companies. The models developed can be used in industry to screen drugs more effectively.

Clinical practise: Since antenatal glucocorticoid treatment is a standard treatment for preterm babies, knowledge generated here regarding potential long-term effects of developmental cortisol exposure on brain development may ultimately inform treatment practices.

Animal welfare: Since wild fish populations are exposed to high concentrations of synthetic glucocorticoids via run-off containing mammalian urine, the new knowledge regarding how glucocorticoids alter fish brain development linked to behavioural abnormalities may inform future policy relevant to animal welfare and aquaculture industry, to prevent pathology and suffering.

Human health: This project will generate fundamental insights into the underlying molecular mechanisms implicated in stress regulation in the brain, leading to behavioural abnormalities including psychiatric disorders. The long-term benefits of this project will be to contribute to the development of therapeutics for stress-related disorders in humans. My research group: The data generated during this project will be used by my research group to inform future studies in the areas of brain aging, gene: environment interactions

and neurogenesis. We will utilise the data to develop new hypotheses and new models of how stress regulates brain development and function linked with behavioural alterations. Molecular targets identified in the project may be utilised to develop new genetic tools and to plan a larger drug screen to comprehensively investigate novel stress regulators.

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

All scientific publications generated as part of this project will be published as gold open access, meaning they will be publicly available. Data sets and code generated will be made available to the community via publicly accessible databases and repositories. New methods generated will be made feely available using open platforms such as protocols.io. Presentation of the work at national and international conferences will facilitate collaborations with other academics in the field.

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.

This project will involve zebrafish at multiple stages across the life course, from larval stages (5 days old) through until aging (36 months old). The effects of stress exposure on behaviour cannot be studied without using animals as this requires coordinated interaction of multiple organs in the body.

Regulations of the stress response, coordinated by the conserved HPA (Hypothalamo-Pituitary- Adrenal) axis consists of highly coordinated interactions between the hypothalamus, pituitary, and adrenal gland, involving multiple hormones that play a role in different parts of the body. It is not possible to culture such a complex system in tissue culture or via organoids. Furthermore, the HPA axis is a vertebrate-specific feature and invertebrate genetic model systems such as fruit fly and worms do not have the HPA axis homolog. This project will use zebrafish at ages across the lifespan and into aging. This is because our aims include investigating the effects of stress exposure on early brain development, as well as the effects on aging. The effects of stress on the brain and behaviour across the lifespan are poorly understood, and these studies will uncover underlying molecular mechanisms that ultimately may be used to develop treatments for stress-related disorders in humans, from early life developmental abnormalities to age-related diseases.

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

Experiments will include:
Animals will be exposed to targeted stress in a more refined manner than has previously been available using a technique called optogenetics, where we are able to increase the animal’s cortisol levels non-invasively by exposing them to blue light, which is part of the normal visible light spectrum. The exposure may occur for a defined period during the lifespan, i.e. early life stress, or may be induced chronically across the lifespan. Stress

may be induced for multiple periods during the lifespan. Control animals will be raised under glass that filters out blue light to prevent cortisol increase.

Animals will be exposed to mild acute stress using established acute stress paradigms such as exposure to a predator stimulus (visual or odour), netting, isolation. Animals may be exposed to multiple stressors in combination, on multiple occasions across the lifespan.

Animals may be genetically modified to induce mutations in genes associated with psychiatric disease and stress susceptibility in humans. Genetic alterations may also be induced in new genes that we identify in our studies as potential regulators of stress-induced adverse phenotypes. Genetic insertions will also be induced in animals to label specific cell types. Genetically altered animals will be used in other experiments, for example acute stress exposure.

Animals harbouring stimulus-activated genetic insertions may be exposed to non-hazardous chemicals or heat, in line with established methodology, to induce fluorescence labelling of specific cell types to be used in microscopy.

Animals will be observed in behavioural tasks, in which we observed the natural swimming behaviour of fish in different scenarios. These might include tasks to investigate social behaviour, basic locomotion, and anxiety. Animals may be observed in multiple tasks, at multiple occasions across the lifespan.

Animals will be used to record brain activity under the microscope. Larvae, up to 14 dpf, will be used in these experiments and will have their head fixed in agarose to immobilise them during the experiment. Animals will be used only once.

Animals will be exposed to compounds to test their effectiveness to ameliorate the effects of stress on the brain and behaviour. Exposure may range from acute (1 hour) to chronic (up to two weeks).

Animals will experience fin clipping or skin swabbing to identify genetic alterations. Fish will be anaesthetised during the procedure (AB) and then housed individually for up to 72 hours to allow for identification of genetic alterations in their DNA. For fin clips a small amount of tail fin (1/8th) will be cut from the fish, whilst for skin swabbing the trunk of the fish will be gently rubbed with a cotton bud. Individuals may be fin clipped or swabbed up to twice during their lifespan.

Animals will be killed via ice slurry to collect brains for downstream analysis including histological analysis of the brain, cortisol analysis and DNA sequencing of the brain tissue.

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

The majority of the animals (>95%) are not expected to show signs of adverse effects that impact significantly on their general well-being.

Animals exposed to stress by increasing their cortisol levels (such as via optogenetic activation) may experience minor long-term effects including mild weight loss (which we routinely monitor), reduced feeding, and minor behavioural changes such as reduced social behaviour as reported in previous studies.

Animals with a mutation in the disc1 gene may exhibit minor long-term effects including

behavioural alterations such as anxiety-like behaviour and altered stress reactivity, as reported in a previous study. Animals with a mutation in the nr3c1 gene may exhibit minor long-term effects including mild behavioural abnormalities as reported in a previous study.

Animals observed during behavioural tasks are not expected to experience any adverse effects since the behavioural tasks involves observation of natural swimming behaviour.

Animals exposed to compounds to test their effectiveness to ameliorate the effects of stress are not expected to experience significant stress. Individuals will experience mild transient stress because of being netted into a container containing the pharmacological substance and being netted out of this container. The incubation in the solution containing pharmacological substance is likely to cause no adverse effects as the treatment is expected to ameliorate the effects of stress. Nevertheless, we will monitor fish for signs of ill health and animals exhibiting unexpected adverse effects will be terminated via a humane method.

Animals exposed to acute stress paradigms will experience mild stress as a result of exposure to a predator stimulus, netting or isolation. However, these stimuli are already experienced by fish in their natural environment or under standard laboratory aquarium conditions, and as such the severity of these exposures is mild and fish are primed to deal with these scenarios.

Animals that experience fin clipping or skin swabbing will experience mild stress associated with anaesthesia and post-surgery recovery. The smallest possible amount of fin will be clipped to maximise recovery time and not induce any changes to normal swimming behaviour. Fish typically return to normal swimming behaviour within 30-minutes of the procedure.

Animals exposed to brain imaging under the microscope will experience a mild stress associated with immobilisation of the head. Larvae may be anaesthetised to minimise discomfort experienced during the experiment. Larvae will be head-fixed and mounted into a specially designed holder to immobilise them and images collected with a special fluorescent microscope to detect cellular activity. Larvae will have their nose and tail free to enable tail movement and sensory experience and will be contained within standard aquarium water at 28.5C to minimise physical discomfort. The duration of the experiment is limited to 1 hour to minimise stress to the 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)?

The severity of protocols 1 to 4 is mild. Up to all animals are expected to be of mild severity, however a significant number will be sub-threshold.

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

- Used in other projects
- 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 aim of this project is to investigate the effects of stress on the brain and behaviour and identify underlying molecular mediators. The effects of stress exposure on behaviour cannot be studied without use of animals as this requires coordinated interaction of multiple organs in the body.

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

I considered cell culture methods, organoids, computational modelling, human studies.

Why were they not suitable?

Regulation of the stress response, coordinated by the conserved HPA (Hypothalamo-Pituitary-Adrenal) axis consists of highly coordinated interaction between the hypothalamus, pituitary and adrenal gland, involving multiple hormones and their receptors. It is not possible to culture such a complex system in tissue culture or using organoids. Whilst simple and discrete aspects of stress regulation can be modelled computationally, it is not yet possible to generate computational models to address complex questions such as how stress modulates behaviour, which involve multiple body systems and include complex elements such as genetic predisposition.

Use of human studies to understand stress regulation in the brain is important, but limited in its ability to control variables and is unable to identify underlying molecular mechanisms in the brain as this requires access to brain tissue. Zebrafish are the ‘lowest’ possible vertebrate to address the scientific aims of this project, and can be considered as a replacement for traditional mammalian 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?

I have used calculations taking into account the typical variation observed in my previous experiments to calculate the minimum number of animals required for this programme of work in order to obtain biologically meaningful and statistically robust results. The number of animals required for breeding has been determined based on previous experience of maintaining a healthy zebrafish stock (10 years experience at 3 institutions).

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

I have utilised the NC3Rs' guidance on experimental design along with their Experimental Design Assistant (EDA) to plan the experimental design, procedural actions, and statistical analysis for this programme of work. This involved incorporating recommendations for randomisation and blinding, determining sample sizes, and selecting suitable statistical analysis methods. The EDA diagram and generated reports will be employed to help plan effective 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 possible, we will use individual fish for multiple purposes. For example, at the end of a behavioural experiment, fish will be terminated, and their brains will be extracted for histological or sequencing analysis, meanwhile their body will be used for cortisol analysis. This reduces the total number of animals required for our 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 study will utilise zebrafish as an animal model to understand stress regulation in the brain. This topic has traditionally been addressed using rodent models, such as rats or mice. The zebrafish is a more refined alternative to rodents, since as a non-mammal they arguably have less capacity for pain, suffering and distress. For many of the experiments proposed, only young larvae are used, which are arguably less capable of suffering than adult fish due to their less developed neural networks. The zebrafish is a widely used model organism for research, and a significant amount of information is already known about its genetics, physiology, and neurobiology.

Because of this we can build on what is already known and put our results into the context of previous studies. Importantly, the stress regulatory system is conserved from fish to humans, and the zebrafish offers enough similarity in terms of genetics and molecular regulators that our results will be relatable to humans. The HPA axis stress regulatory system does not exist in non-vertebrate species, so it would not be possible to use invertebrate model organisms such as worms or flies for our study.

Because our aim is to understand how stress affects the brain and behaviour, we cannot use simple tissue culture, advanced 3D cultures or single organoid systems or rely on computational modelling to understand such a complex phenomenon.

In this study we will expose animals to stress using a technique called optogenetics, where we are able to increase the animal’s cortisol levels non-invasively by exposing them to blue light. This is the most refined way of inducing stress, as it removes the animal’s sensory experience of perceiving and anticipating a stressor. Traditional rodent models initiate stress in young animals by removing the pups from their mother. This paradigm

induces clear distress in both the pups and the mother and has significant effects on the health of the pups including poor growth and weight loss due to the lack of proper nutrition and fragmented maternal care during early life. In the optogenetic zebrafish model of stress, animals exhibit no detectable signs of distress and are indistinguishable from non-stressed fish.

In our studies animals will also sometimes be exposed to acute stress using established acute stress paradigms such as exposure to a predator stimulus (visual or odour), netting, isolation. These stimuli are already experienced by fish in their natural environment or under standard laboratory aquarium conditions, and therefore the fish are already primed to deal with these scenarios. Fish experience netting during routine handling and will be netted for no more than 30 seconds. Fish also experience isolation during routine experiments for purposes of identification (such as fin clipping). In the wild, fish are exposed to natural predators daily, thus exposure to a predator stimulus in the form of a visual cue or odour is within the normal experience of the fish.

Animals will be observed in behavioural tasks, including social behaviour paradigms, locomotion assays, anxiety tests. During these tests we passively record the natural swimming patterns of either individual fish or groups of fish in a tank. Animals observed during behavioural tasks are not expected to experience any adverse effects since the behavioural tasks involve observation of natural swimming behaviour.

To induce fluorescence labelling of specific cell types, animals harbouring genetic insertions may be exposed to non-hazardous chemicals or heat. The chemical used is designed specifically to have no effect other than to induce the fluorescence labelling of the cells of interest. In cases where heat is required for induce labelling of cells in animals, the swimming water of the fish is briefly (for 30 minutes) heated to 37C. This is a standard protocol performed for this purpose. Zebrafish are a tropical fish and can tolerate a temperature range of 10.6C to 41.7C. As such, the exposure of fish to 37C for 30 minutes is within their natural tolerance.

To measure brain activity, zebrafish larvae, up to 14 dpf, will have their head fixed in agarose to immobilise them, during which brain activity is recorded under the microscope.

For these studies, only young larvae are used, which are arguably less capable of suffering than adult fish due to their less developed neural networks.

Larvae may be anaesthetised to minimise discomfort experienced during the experiment.

Larvae will have their nose and tail free to enable tail movement and sensory experience and will be contained within standard aquarium water at 28.5C to minimise physical discomfort. The duration of the experiment is limited to 1 hour to minimise stress to the animal.

Animals will be exposed to compounds to test their effectiveness to ameliorate the effects of stress on the brain and behaviour. Exposure may be acute (1 hour) or chronic (up to several weeks). The compounds are chosen due to their potential as therapeutics and no adverse effects are expected.

Exposure will occur via the swimming water of the fish and thus the procedure is non-invasive. For long-term exposures, fish will be exposed in groups, rather than individually, to ameliorate any anxiety during the procedure.

To identify genetic alterations, animals will experience fin clipping or skin swabbing to collect DNA. Fish will be anaesthetised during the procedure, to minimise pain and distress. For fin clips the smallest possible amount of tail fin (roughly 1/8th) will be cut from the fish, which quickly grows back. Fish are swimming normally again within 30 minutes of the procedure. Where possible, skin swabbing will be used as an alternative method to collect DNA. In this case the trunk of the fish will be gently rubbed with a cotton bud, which is less invasive than fin clipping. For skin swabbing, fish are not anaesthetised, but are temporarily restrained out of the water. Recent research suggests that this technique may be less stressful to the fish compared to fin clipping.

To collect samples for downstream analysis including histological analysis of the brain, cortisol analysis and DNA sequencing of the brain tissue, fish will be killed via ice slurry.

Recent studies have indicated that this is a more humane method for euthanasia of fish compared with the traditional paradigm involving overdose of anaesthetic via immersion in MS 222. These studies found that fish showed fewer signs of distress and died faster via ice slurry compared with MS222. Immersion via ice slurry is a standard approved method for termination of fish in the USA, Australia and in parts of Europe. This method was also approved on the PPL under which I conducted my post-doctoral studies at a different institution. This is a more scientifically robust method to achieve our aim because it does not activate the neuroendocrine stress response, does not involve exogenous chemicals, and slows down metabolism to allow us to live capture cell states more accurately.

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

The HPA axis stress response system is a vertebrate-specific feature and invertebrate genetic model systems such as fruit fly and worms do not have the HPA axis homolog.

This project will utilise zebrafish at ages across the lifespan and into aging. This is because our aims include investigating the effects of stress exposure on early brain development, as well as the effects on aging. As such, many of the experiments will utilise larvae prior to the onset of protection.

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

Animals that are exposed to stress or fin clipping, and new genetically altered zebrafish lines will undergo increased monitoring to detect any adverse effects more quickly. Ageing animals will be carefully monitored by trained staff trained to monitor for any adverse age-associated phenotypes. Group sizes in aging experiments will be increased to accommodate for loss of animals. If adverse effects are observed, animals will be treated accordingly, and animals that develop severe effects will be humanely killed. For fin clipping, peri-operative analgesia will be used, following guidance from the NVS, to minimise pain to the animals.

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

I will follow best practice guidelines published by the NC3Rs such as their guidance document on zebrafish welfare which includes information on Housing and husbandry; transportation and reception; breeding and identification. I will follow the best practices published by the RSPCA in their guidance document ‘Guidance on the housing and care of Zebrafish’. I will adhere to the PREPARE guidelines for planning animal research and

testing.

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

I receive the NC3Rs newsletter to keep up to date with developments in the 3 R's.

During the project I will attend relevant 3 R's symposia. I will attend scientific conferences relevant to the project to learn about techniques and approaches used by others that may aid the 3 R's aspects of this project.

29. DNA Damage Responses 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

neurological disease, DNA repair, PARP enzymes, DNA breaks

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 elucidate the molecular mechanisms by which DNA damage results in genome instability and 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?

This work has the potential to identify and develop novel therapeutic strategies, and ultimately treatments for human diseases associated with unrepaired DNA damage.

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

The outputs of this work will be increased knowledge of the links between defects in DNA repair and human disease and, based on this knowledge, the identification of possible new therapeutic opportunities. As with my previous licence, on this same topic, key outputs will be published in peer reviewed international journals, including high impact journals such as Nature, Cell, and Science.

While the general scientific question is the same, this PPL will focus on the molecular defect in neurons that underpins the defect in cytosine demethylation and calcium homeostasis we identified during the current PPL.

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

The primary beneficiaries the are relevant scientific community, who will benefit and inform their own experiments based on our studies. In addition, key beneficiaries are also clinicians and their patients with rare genetic diseases associated with defects in the repair and response to DNA damage, of which there are currently thousands, world-wide. For example, I manage a diagnostics activity in which we empoy molecular tests to identify disease-causing defects in DNA repair in patient-derived samples provided by clinicians. The work conducted under my existing project licence, and the current application, thus informs and is informed by, clinically relevant analyses.

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

Both successful and unsuccessful outputs of this work will be disseminated at leading international conferences, in high quality/high impact publications (in line with ARRIVE & PREPARE guidelines), and through enhanced and extended collaborations with other research groups, clinicians, and the pharmaceutical sector.

Species and numbers of animals expected to be used

- Mice: We anticipate using a maximum of 17,750 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 required for experiments where the impact of DNA damage on human disease is under investigation, because these are the least sentient organism that recapitulates the complexity observed in humans in DNA damage responses, and the disease pathology that arises if such responses are detected. The use of mice is thus critical to our understanding of how DNA damage induces human disease, and to identify new therapeutic opportunities to cure such disease. Most of our experiments will employ neonatal mice (postnatal day 0-3) to prepare brain tissues or cells for in vitro analysis, or juvenile mice rather than adult/aged mice to minimise the adverse effects of accumulated DNA damage.

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

Mainly, we will breed mice to compare mutant and normal littermate controls, or where possible cells (e.g. neurons) derived from these animals at postnatal day 0-3, to address the impact of the mutated gene in question on health and disease. In some cases animals may be treated (orally or by injection) with potentially therapeutic compounds to identify possible cures for DNA damage-associated diseases. When rederivation is required, unmodified or modified embryos may be developed to term by surgically placing them into a female rendered pseudo-pregnant by mating with a surgically vasectomised male. During the course of the project, if at any stage an animal experiences poor health that

cannot be ameliorated, it will be killed humanely and in a timely manner. All animals that have reached the end of their study will be killed.

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

Most animals (>95%) will experience mild or no discomfort and any discomfort will be transient with no long lasting harm. Some animals (<5%) will lack Xrcc1, and may experience moderate discomfort associated with DNA damage related phenotypes (e.g. ataxia). Note that while ~50% of the animals employed in this PPL will be part of a moderate protocol (Protocol 5), most of the animals in this protocol will be unaffected heterozygous animals for breeding or wild type/heterozygous offspring, and thus will experience no discomfort. Mice will be monitored frequently for health and humanely killed if their health becomes poorly and/or the humane endpoint is approached. When in doubt about the health of an animal, advice will be sought from the Named Veterinary Surgeon (NVS).

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 with mild or no discomfort: >95%

Mice with moderate discomfort: <5%

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

- Used in other projects
- 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?

Mice are required for experiments where the impact of unrepaired DNA damage on disease-related phenotypes is under investigation. These questions are critical to our understanding of how DNA damage induces neurological disease.

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

For most experiments (>95%) we use human cell lines in a test tube for our experiments, including nerve cells. This limits the need for live animals.

Why were they not suitable?

For some experiments the use of animals is unavoidable e.g. to test our idea that preventing activity of the protein PARP1 reduces the neuropathology in animals lacking the DNA repair protein XRCC1.

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 estimated here are based on our average actual usage during our current project, factoring in that the new project is significantly smaller than the current project. There is a significant reduction (~25%) in estimated mouse usage, compared to my current project licence. This is because we are now employing more in vitro model systems such as human iPSC cells and more ex vivo techniques (e.g. neural cell cultures and electrophysiological approaches using brain tissue derived from the mice).

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

As indicated above, there is a ~25% reduction in estimated mouse usage, compared to my current project licence. This is because we are now employing more in vitro and ex vivo techniques, which has reduced the number of experiments requiring live mice.

In addition, where live mice are required, minimum group sizes for experimental mice will be determined by standard power analysis (e.g. the NC3R EDA). We will also minimise mouse numbers by designing paired analyses that measure several parameters simultaneously (e.g. measuring the impact of a particular gene loss on multiple cell types in multiple tissues), allowing us to reduce the number of both test and control animals.

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

Where required, mice will be kept at the minimum number required to maintain relevant colonies and conduct experiments. Typically, we maintain colony sizes of 4-6 breeding pairs per strain, and when not needed these strains are cryopresevered to prevent needless breeding programmes.

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 DNA repair defective mice in this project, to understand how DNA damage causes human disease. Most animals (>95%) will be used to generate brain tissue and/or

cells for use ex vivo, thereby minimising the number of live mice and thus suffering. Some experiments (<5%) will involve the measurement of wobbly gait/movement (ataxia) in live mice, which is a measure of neuronal death or dysfunction in a part of the brain known as the cerebellum. These animals will be used for only short periods and then killed humanely before postnatal day 15, thereby minimising their experience of ataxia. The maximum severity these animals will experience is a moderate phenotype (ataxia).

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

Animals that are less sentient than mice do not possess the complexity in DNA damage response that is present in humans. For example, the protein sequence and function of mouse XRCC1, which is the major focus of this project, is very similar to human XRCC1, whereas the protein sequence and function XRCC1 in less sentient animals (e.g. flies, fish) is not. This means that less sentient animals are not good models for XRCC1-defective human disease.

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

Under all circumstances we minimise animal suffering by frequent (e.g. daily/twice daily) health and behaviour monitoring, including the use of health score sheets, and by humane killing of animals if the humane end point is approached. Appropriate pain management and post-operative care is in place, as detailed in each of the relevant protocols. Under all circumstances, if in doubt, advice will be sought from the Named Animal Care and Welfare Officer (NACWO) and Veterinarian (NVS).

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

We will utilise a variety of online, published, and local sources to inform our experiments including the previous scientific literature, web resources such as the NC3R Experimental Design Assistant, and LASA for good surgical technique.

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

I am in close contact with the NACWO and NVS, who regularly inform project and personal licence holders of recent developments in the 3R's, including frequent appraisal of 3Rs-associated sites such as NC3Rs & FRAME. This allows me to discuss and implement appropriate refinements as and when appropriate. For example, after discussion with the NACWO and NVS, we implemented a management strategy for Xrcc1-defective mice that ensured that they do not experience more than a moderate phenotype, by humanely killing these animals before their juvenile onset of sporadic seizures (P15). Instead, to study their seizure phenotype, we use ex vivo brain slices in which we can recapitulate their seizure phenotype in vitro by incubating the brain slices in epileptogenic buffer.

30. Polyclonal, Biologicals, Normal Serum & Antigen Production

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
  - 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

Life Saving, Disease Testing, Blood Safety, Cancer Research

Animal types	Life stages
Rabbits	adult
Sheep	adult
Goats	adult
Alpaca	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 produce Antibodies and Antigens for use in;

1. Medical Diagnostic Test Kits & Systems for the rapid detection of disease in Man

2. Basic Research into Disease conditions e.g. 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?

The real value of Medical Diagnostic Kits and Test systems is the rapid detection of infection so that appropriate treatment can be given immediately, in many cases this is can be life-saving e.g. The rapid Detection of Bacterial Meningitis in children.

Testing of donated blood for a range of diseases is Important for several reasons;

1. Prevent the spread of disease e.g. Hepatitis B & C and Syphilis

2. Maintain public trust and confidence- Testing for diseases reinforces the integrity of blood banks and ensures blood is safe to use.

3. Regulatory Compliance - A legal requirement

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

The Antibodies & Antigens produced from this project support Gold Standard testing and Research across the Globe.

The Outputs are mainly products used for routine testing but will include the redevelopment of some tests and vaccines to ensure they continue to improve patient outcomes by accurately diagnosing and monitoring disease, this includes early detection and prevention of certain diseases.

Specifically some diseases e.g. Syphilis, continue to be a world-wide problem with a record number of cases, screening requires high grade antigens for increased early stage testing to ensure prompt treatment.

Research outcomes include improved understanding of disease including causes, risk factors, prevention strategies and treatment options.

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

Many groups will benefit from this project in both the short real time term (Disease Testing) to Long term (Research) outputs.

Short term benefits - Immediate - will be experienced by Individual patients, Healthcare providers and Public Health Agencies.

Long term benefits 0-5years will be seen with those working on Research projects in Academia and Scientific Communities, further on beyond the time of this project Health Organisations and Medical Professionals may benefit from contributions of knowledge for the development of new drugs and therapies.

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

The Business is committed to using its AWERB group to maximise the outputs in terms of antibodies & antigens by reducing animal use and refining processes / improving Welfare.

The Business has a solid track record with 3R initiatives, this is achieved by engendering a strong welfare culture within all members of its organisation and customers, clients are encouraged (during visits, audits and routine communications) to support the ongoing work of the AWERB group

Knowledge of some of the techniques used and Care routines are often shared with other groups / organisations by collaboration.

Species and numbers of animals expected to be used

- Rabbits: 7500
- Sheep: 850
- Goats: 1150
- Camelids: 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.

Adult Sheep, Goats and Rabbits are typically used for the production of antibodies, the main reason for the choice of these species is;

The animals share the same fundamental class as man - Mammalia, the animal species stated are in plentiful supply, originally chosen for their ease of use and ability to produce high quality antibodies.

Specifically sexually mature male rabbits (minimum 26 weeks of age) are the only / primary animal host for the study and large scale production of Treponema pallidum bacterium for several reasons including their susceptibility to Treponema pallidum infection, their relatively low cost compared to other animal models, and their similarity to humans in terms of immune reaction.

Camelids, such as llamas, and alpacas are used as they produce a unique type of antibody known as single-domain antibodies or nanobodies. These antibodies are significantly smaller than conventional antibodies found in humans and other mammals, making them attractive for various biotechnological and medical applications due to their high specificity, affinity and stability making them a powerful and versatile tool in biotechnology and medicine, with the potential to revolutionize various fields through their unique properties and applications.

Adult animals are typically used for antibody production because they have a fully developed immune system, which allows them to mount a robust immune response to the antigens used to elicit the production of antibodies. Additionally, adult animals tend to produce larger quantities of antibodies compared to younger animals, making them more efficient for antibody production purposes.

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

<u>Antibody Production</u>

Animals used for antibody production will be dosed with antigen then blood sampling will take place for the purpose of assessing antibodies.

Antigens will be administered either under the skin (Subcutaneously) - Sheep Goats Camelids Rabbits or Intravenously (for bacterial antigens) - Rabbits.

Animals are monitored at all stages of the processes to limit adverse effects, dosing is reduced or omitted if there is a concern that further inoculations may cause distress to the animal.

Once a sufficient antibody response has been achieved animal's blood will be harvested under terminal anaesthesia and further processed.

Syphilis Antigen Production

Rabbits will be used for the production of syphilis antigen. This will involve a single administration into the testicle on one occasion.

Animals will be monitored twice daily from Day 7-14 for inflammation of the testicles (orchitis) at which stage the animals will be killed under terminal anaesthesia when the testicle will be harvested for processing.

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

Animals may suffer reactions at injection sites and bruising at sites of blood sampling, these are rare and short lasting.

Rabbits used for some intra-venous injections of bacterial antigens will suffer mild flu like symptoms which will last no longer than 8hrs, these reactions are limited to the first week of dosing, subsequent injections will produce less adverse effects as the animal builds immunity to the antigen.

In the majority of cases rabbits used for T.pallidum production will develop swollen testis, this is an expected reaction lasting no longer than 7-10 days during which time animals will be closely monitored by twice daily checks on each individual animal, in rare cases animals may lose weight, in this case they will be terminated before severe 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)?

Sheep - Mild Severity

Goats - Mild Severity

Camelids - Mild Severity

Rabbits - 33% Mild / 66% Moderate Severity

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?

There are currently no alternative methods to produce the class of antibodies or syphilis antigens required for the test systems supported by the Business used to detect disease in Man, the same can be said for antibodies for the unique research projects for which we offer a service.

Sensitivity and specificity are the two key measures of a diagnostic test's ability to accurately detect the presence or absence of a disease or condition, sensitivity refers to the proportion of true positives among individuals who actually have the disease, specificity on the other hand refers to the proportion of true negative results among all individuals who do not suffer from the condition or disease, there is often a trade-off between the two in a given test system.

Test systems falling below required standards are often legally removed from the market (recall notice) by regulatory bodies where the test has been carried out, it is important for a low rate of false negatives and false positives, the rapid performance agglutination kits supported by the business are extremely robust with a 99.33-100% specificity and a turnaround time of under 30 minutes which is crucial in certain disease conditions where appropriate treatments (led by the test result) can be given immediately. e.g. bacterial meningitis

Agglutinating test systems are supported by animal derived antibodies which out-perform all alternative synthetic antibodies, these tests are also very important as they require very little laboratory Infrastructure or training and can therefore be carried out in countries the world over.

Customers requiring the antibody services of the business are required to submit a detailed 'Antibody Services Questionnaire' for each and every antigen requiring the use of animals, the submission of this document is required for each occasion and every antigen regardless of previous submissions.

The Questionnaire is scrutinised by the Certificate holder, Project licence holder and at least one senior licensee of the business, the Named Veterinary surgeon (NVS) may be consulted at times to ensure the application meets all the conditions of the licence.

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

Non animal derived antibody alternatives have been considered and are used where possible,

e.g. Recombinant and synthetic antibodies, antibody mimetics and aptamers

Non animal derived production of Treponema pallidum (Nichols strain) using culture media has been considered.

Customers requiring the antibody services of the business are required to consider non-animal alternatives, this is a mandatory section in the 'Antibody Services Questionnaire' customer responses will be examined carefully during the application process.

Why were they not suitable?

Animal derived antibodies differ from the non-animal alternatives in a number of ways;

1. They have a complex structure which recombinant and synthetic antibodies cannot mimic

2. Animal derived antibodies perform functions in the immune system that non animal alternatives do not match

3. Animal derived antibodies are highly specific (crucial for the diagnostic systems supported by the business) non animal alternatives have reduced affinity which if used in test systems could lead to high levels false positive results and potential removal of the test from the market.

Treponema pallidum is an incomplete anaerobic non spore forming motile bacterium that requires an exacting environment of the correct medium, temperature and oxygen concentration to reproduce, efforts to use this non animal approach have proven unsuccessful for the volumes of bacterium required to support the test systems currently used.

Customers requiring the antibody services of the business are required to check Immune libraries, naïve, semisynthetic and synthetic libraries as these are an important source of antibodies, this is a mandatory section in the 'Antibody Services Questionnaire' customer responses will be examined carefully during the application process.

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 based on a combination of;
- Known contracted work
- Potential New projects
- Site Capacity

For new antigens 1 or maximum 2 animals are used, for bulk production the number of animals used takes in account response rates in the injected population and titre levels.

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

The business will discuss animal use with each customer as part of its management review, clients are encouraged to use larger species wherever possible to reduce the number of animals used, this is especially important where new products are under development however there are limitations using larger species such as a requirement for B-cells from splenectomies where smaller species are more suitable, some bacterial antigens will naturally evoke stronger responses in smaller species.

Communication with clients during each project application is key to the reduction of animals, the main areas of discussion will focus on antigen dosing where the use of appropriate single carriers, or multiple carriers for the same antigen can illicit strong antibody responses leading to a reduction in animals.

Careful use of adjuvants and the use of different strains of rabbits will potentially have a positive impact on reduction during the design stage of each piece of work.

The experimental design for T.pallidum is constantly evolving to optimise the harvest of antigen and reduce the number of animals used, the business will strive to refine their methods to enhance the efficiency of antigen harvest from rabbits.

We have considered and tried challenging animals with multiple antigens simultaneously this approach has proved unsuccessful due to cross reactivity, on absorption no specific antibodies were harvested.

Goats are particularly bad hosts for multiple antigen stimulation, we space their routine health prevention vaccines from boosters for scientific use for this reason.

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

The main steps involved in reducing animals is to source or breed animals that produce higher volumes of blood due to size / weight.

This can be readily achieved;

Sheep; an in-house breeding programme using the largest known breeds available (Wensleydale / Leicester Long Wool rams crossed with a docile robust good mothering ewe e.g. Dorset Horn) peak weight of offspring are often up to double that of standard Mule sheep.

Rabbits; Giant type breeds peak at 8kgs, double the standard purpose bred New Zealand White (NZW) rabbits that have historically been used in the Science Industry. NZW rabbits are a poor choice for Antisera and Treponema pallidum production and were originally used in the 20th Century due to their availability from the meat Industry.

Giant type rabbits carry less fat and more muscle which adds to the advantage of volume of blood harvested on termination. There is a massive reduction of over 50% animal use using Giant breeds compared to Standard NZW strains for antisera production.

Giant breeds are typically more docile and easy to handle compared to the NZW strain which can be aggressive and less easy to handle.

The case for using Giant rabbits for T.pallidum production is even more compelling, typical yields of 52- 60mls of finished product per rabbit compared to 15mls if using Standard NZW strain, a huge reduction of animal use.

The use of ex-breeding buck rabbits from all breeds of rabbits further increases the yields of T.pallidum by up to 20%, rabbit suppliers do not have the capacity to supply ex breeding bucks in the numbers used for this procedure, sourcing rabbits from specialist breeders in the UK and EU will allow access to a wider range of breeds in the numbers required by this procedure to meet further reduction initiatives.

Goats; - castrated male goats which would normally go to slaughter will typically achieve higher peak weights than females and can also fulfil this objective.

Environmental conditions; creating an environment to mimic natural breeding conditions of the male rabbit further reduce animal use this is achieved by controlling light cycles, 16hrs light, 8 hours darkness using lighting systems which mimic dawn dusk (crepuscular)

zones, breeding conditions are further enhanced by controlling a stable temperature of 12 degrees C (+/- 2 degrees C)

In summary the approaches used for sourcing / breeding animals listed reduces numbers by up to 50% for blood related products due to increased harvest of blood (serum) and reduces animals for treponema production by up to 75%, environmental conditions play a key role.

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 will use Sheep, Goats, Camelids and Rabbits and are the best choice of species for the Project due to the ease of providing safe and comfortable housing / environment to ensure the least suffering and distress. Procedures used for the purpose of producing antibodies are minimal in terms of severity to achieve the objectives.

Sheep, goats, and camelids will not suffer lasting harm, those that do not exhibit a sufficient sustained antibody response or have experienced a decrease in antibody titres due to age may undergo a health evaluation by the NVS and to join a conservation grazing herd for the rest of their natural life, grazing is often the best way to stimulate the growth of a wider variety of plants and animals, some of our retired animals have already encouraged the growth of rare plants which are of national interest.

Using larger animals such as ruminants and pseudo-ruminants allows for larger harvest and therefore a reduction of animal numbers.

This range of species, specifically camelids, allows production of antibodies of different shapes with other biotechnical applications.

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

The animals listed have been historically used for the purposes of this Licence for several reasons;

Less sentient animals, such as insects and bacteria, are not commonly used for antibody production because their immune systems are not as complex as those of mammals. Mammals have evolved a highly sophisticated immune system that is capable of producing a wide variety of antibodies in response to antigens. This diversity and specificity of antibodies are crucial for various applications, such as disease diagnosis and research. Additionally, mammalian antibodies are more similar to human antibodies in structure and function, making them more suitable for use in biomedical research and diagnostic applications. In contrast, antibodies produced in less sentient animals may not be as effective or reliable due to differences in their immune systems and antibody structures. Furthermore, the use of animal mammals allows for large-scale production of antibodies due their size and scalability.

Employing less sentient animals than rabbits for syphilis antigen (T.pallidum) production is not possible, this genus of bacteria can only be propagated in rabbits, the life stage for this process is critical, animals need to be fully grown mature male rabbits for the bacterium to replicate in the numbers required to provide enough antigen for diagnostic testing.

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

Refinements will be driven by the AWERB group where all members are expected to contribute to plans and trials to minimise the welfare cost to the animal, a recent excellent example of this in the Business was a feeding trial where 5 feed supplements were given to groups of rabbits with the aim to aid stress related digestion problems during syphilis antigen (T.pallidum) production and enrich the diet of the animal. The feeding of fibre sticks emerged from the data as a clear winner in terms of minimising the welfare cost. This is now part of the standard feed regime for the species.

During the course of the current Licence the Business has replaced 70% of small animal buildings with state of the art facilities, the AWERB group were active in the design of this project, rabbits are now housed in purpose designed buildings with air conditioning which operates at +/- 2 degrees C at a temperature where the animals feel most comfortable which is typically in the range of 12-16 C for rabbits.

A lux controllable LED light source with controllable light cycles and natural dawn dusk cycles complements the environment to provide a natural stress free habitat, the buildings are complemented with the latest single or group cages with clear plastic panels so animals can see other animals at all times, the cages allow natural behaviour and include a shelf and area of seclusion. Routine feed includes a high fibre pellet, fresh irradiated hay and extremely palatable fibre sticks, fresh filtered UV treated water is provided daily in a one litre bottle that allows easy measurement of daily consumption, environmental enrichment includes but is not limited to play tunnels and cardboard.

Sheep are housed in fields which include shelter, Goats will be housed in-doors but have access to open fields.

With regard to refinements associated with procedures (injections and blood sampling) animals are monitored and reactions recorded at 5 minutes, 2 hours, 4 hours and 24 hours after each procedure, injection site maps are recorded for all primary injections for each animal, animals are regularly weighed including prior to all procedures, antigen injections may be omitted or reduced at any time if the Licence holder feels that this decision is in the best interest of the animal. Protocols are maintained for each animal where data collection (injection / bleed volumes, animal weight, site maps, procedure checks) are maintained by each Licence holder at the time of the procedure, the data can be used to further refine the antibody process by providing a base line / point of reference for each animal to reduce potential harm.

Individual Licence holder's abilities to carry out procedures (injections and blood sampling) are assessed by the Project Licence holder on an ongoing basis, whilst training plans are issued annually retraining may take place at anytime, the Named Veterinary Surgeon will often be involved in this process.

The AWERB group will continue with the objective to refine the overall well-being of the animals under their care.

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

The Business adopts best practices from a variety of sources principally from the ASC & NC3Rs, specifically to adhere to the principles of the 3Rs, the AWERB group of the business strives to be active and innovative in this respect, key to the principle of operating in the most refined way is to plan each project to discuss potential issues at an early stage, this is carried out using a customer questionnaire which is issued prior to all projects so that the business understands the work and can discuss potential non animal alternatives with the customer and avoid potential replication of work, at this stage the business will discuss reduction and refinement policies within the customer.

The business works on a 'Plan, Do, Document, Review' basis, whilst the nature of the work follows standard protocols there are common areas that the business's AWERB group will focus on such as the use, reduction or replacement of adjuvants and the reduction of protocol duration.

All of these goals are an ongoing process, sharing successful refinements with other Veterinary Surgeons and Research groups is key to this process.

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

The Business will keep informed of the latest advances via;

Frequent Literature searches in advance of AWERB meetings that in this Business will focus mostly on the 3 R's

Frequent discussions with the NVS

Meeting and Bench marking within the Industry

31. Targeting endoglin function in cardiovascular 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

Endoglin, Cardiovascular, Hereditary Haemorrhagic Telangiectasia (HHT), Preeclampsia, Genetics

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 understand how human mutations in endoglin gene and changes in endoglin function cause or contribute to different cardiovascular diseases, and to apply such knowledge for therapeutic application.

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 project studies the function of a protein called endoglin, also abbreviated as ENG. It locates mostly on the surface of the cells lining the blood vessels. Changes in ENG

function have been implicated in two vascular disorders, namely, Hereditary Haemorrhagic Telangiectasia (HHT) and preeclampsia.

HHT is a genetic disorder where the blood vessels, particularly capillaries, are not properly developed, resulting in distorted and weak connections between small arteries and veins which can lead to regular bleeding. HHT patients typically have red blood spots on the skin (telangiectasia) and regular nosebleed that may require blood transfusion, but it is the bleedings in the internal organs that are serious and can cause life-threatening haemorrhage. In addition to bleeding risk, many patients have distorted blood vessels in the lung, brain and liver, affecting the function of these essential organs.

Type 1 HHT (HHT1) is caused by inherited mutations in ENG gene which lead to cells making less or faulty ENG protein, whereas HHT2 is caused by mutations in a gene called ACVRL1 which interacts with ENG. Mutations in ENG and ACVRL1 account for over 80% of all HHT patients. Average rate of HHT occurrence is 1 in 5000-10000 of the population worldwide.

Preeclampsia is hypertension (high blood pressure) during pregnancy and can be very dangerous for both the mother and the baby. During pregnancy, cell surface ENG is significantly increased in the placenta, and a small proportion of this can be cut and release a large fragment into the blood known as soluble endoglin, or sENG. Levels of sENG, along with another protein called sFlt1, are elevated by more than 10-fold in women with preeclampsia compared with those without. Current literature suggests that elevated levels of sENG and sFlt1 may directly cause preeclampsia, but more studies are required to confirm this.

There is no cure for either HHT or preeclampsia, and we still don’t know how the changes in ENG function cause these two vascular disorders. The aim of this project is to apply what we have learnt about ENG function at protein and cellular levels and perform further studies in mice to understand whether ENG changes are present in mice, whether they contribute to the development of HHT and preeclampsia, and whether we can apply novel therapeutics to prevent and reverse the diseases. Such knowledge may help us find novel information in the blood proteins (called biomarkers) that can predict or monitor these disorders. It may also help to develop molecules that could restore the function of ENG as novel therapeutics.

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

This project has three objectives.

The outcome of objective 1 will reveal whether and how increased levels of sENG cause or contribute to the development of preeclampsia. It has been known for a long time that serum levels of sENG, along with sFLT1, are significantly increased by more than 10-fold in women with preeclampsia. The impact of such changes on the development of preeclampsia is not fully understood and our studies will reveal important insight into this. Such knowledge could lead to either new treatment targets or novel biomarkers for early detection of preeclampsia.

The outcome of objective 2 will be the results of testing novel therapeutics in mouse models of HHT1 – such mouse models mimic the human loss of ENG gene and mice develop some clinical symptoms similar to human HHT1 patients. Our potential novel therapeutics target the genetically identified pathway, aiming to correct the underlying cause of HHT1.

Objective 3, along with objective 1 and 2, will provide essential knowledge on ENG function at more detailed levels, for example, how endoglin interacts with different proteins and pathways in the blood vessel cells to affect blood vessel function. Such knowledge is crucial for a comprehensive understanding on how ENG achieves its normal function in the blood vessels and how the changes in its function contribute the two devastating vascular disorders.

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

In the short term, the knowledge gained from this project will be published in scientific journals and benefit other medical researchers. Such research will provide further information on how our body system controls blood vessel functions, thereby develop better therapies for the treatment of preeclampsia and HHT. In the middle to long term, such advances in PE and HHT research may lead to novel biomarkers and therapeutics, which will help physicians to better manage and treat patients, and provide benefit to the patients as well as their families, and our society as a whole.

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

We will disseminate our research findings timely and broadly, at scientific conferences and in scientific journals. We will make connections with patients' group whenever possible to explain our research. If any findings that can lead to a potential medical product, such as potential novel therapeutics, we will work with relevant drug discovery partners and translate our findings in a timely manner to provide patient benefit.

Species and numbers of animals expected to be used

- Mice: 6150

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 objective 1, we study preeclampsia which is high blood pressure during pregnancy that is resolved upon delivery, therefore we will need to use pregnant mice for the study. To determine whether the phenotype we observed is specific to pregnancy or not, we will need non-pregnant female and male mice as controls. It is not possible to use lower species animals for this purpose because we want to recapitulate the disease during the pregnant stages in human which are not present in lower animal species. It is essential to use a mammal and a mouse is the smallest, reliable mammal for this research.

For objective 2, we are modelling a human heritable blood vessel disorder HHT, manifested as mal- formed blood vessels in different parts of body. HHT patients are mostly adult, and many current literatures use new-born mice. We use a genetic modified mouse line that carries the hidden mutation in the gene that cause human type I HHT. We can allow the mice to grow to adulthood completely free of disease, and only induce the disease by giving an inducing agent called Tamoxifen which will delete the target gene and then mice will develop the disease symptoms over the following 5 weeks. It is this time window we will focus our research to study the pathological change and the resolution by potential new therapeutics.

For objective 3, we use these mice to help our research in understanding why the gene mutated in HHT can cause HHT-like diseases. These mice carry the hidden mutations that do not cause any disease in young mice less than 4 months old. We can generate cells from any organ and delete the HHT-disease related genes in cells isolated from different tissues after mice are euthanised, therefore we will not cause any pain to any live animals.

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

Injections and procedures will be kept to a minimum to achieve the required results. Tests and small- scale studies will be performed on new molecules and drug candidates to determine dose levels, dosing frequencies and the most appropriate route of administrations required to assess the effect of the molecules. We will also regular review published results and modify our experiment accordingly when required.

Animals will be kept under anaesthesia for the minimum time possible to achieve the required results. Sometimes we need to measure the blood pressure in the heart chambers and lung circulation as well as heart functions, as these are part of the disease symptoms we are trying to establish in the animal model. We use a method called catheterisation where we put an electronic probe into the heart under general anaesthetic condition. Typically, the catheterisation procedure takes approximately 30-40 minutes to complete. While under anaesthetic animals will be maintained at an appropriate temperature by using a heat mat or similar heating device. Animals will be euthanised at the end of catherterisation experiment.

Substances injected will be of known safe dose concentrations and administration routes. The administration of substances 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. Test molecules will be dissolved in solutions that have been tested and deemed safe to use.

Blood sampling via superficial veins (usually from the hind leg) will be kept to a minimum and safe volumes calculated based on estimated circulating volumes.

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

Genetically altered mice produced under this protocol are not expected to exhibit any harmful phenotype. Some animals will experience mild transient pain associated with an injection or blood sampling procedure.

For the two disease phenotypes we are modelling in this application, HHT and preeclampsia, animals might experience some human disease-like symptoms. For example, in HHT model, mice may develop enlarged hearts and weak blood vessels which can cause unexpected bleeding. For mice modelling preeclampsia, they may develop high blood pressure, proteins in urine samples and reduced placenta size.

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 50%

Moderate 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?

The animal research in this project is essential to our understanding of several human vascular disorders including HHT and preeclampsia. We have performed over 10 years of research using non- animal alternatives, such as using proteins and isolated cells, to come up with the hypotheses and objectives to be tested. This project focuses on the disease model set up and testing therapeutic intervention. In order to translate our basic science research to patients' benefit, disease model studies using animals are an important step that cannot be replaced before clinical applications.

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

We will use non-animal alternatives whenever possible. We have used and are still using recombinant protein technology and structural biology to understand how ENG and its interaction partners achieve their functions under normal circumstances, and how mutations found in patients cause changes to protein functions. We also use cellular models to study how different proteins interact with each other inside and at the surface of the cells to influence the overall function of the cells. In collaboration with others, we will use patient samples and advanced cellular models whenever we can.

Why were they not suitable?

Pathological changes in human diseases are never limited to a single tissue or a single cell type. The disease-causing mutations in ENG affect blood vessels in multiple organs. To understand whether such changes are the cause or the result of a disease is crucial for clinical application. Non-animal alternatives will not be able to model the interactions between different tissues and organs.

Furthermore, the impact of normal physiology, such as sex and age, during the disease onset and development cannot be tested without using animal models. Carefully designed animal studies allow us to detect the pathological changes in multiple organs simultaneously and over a period of time, thus we can test the effects of potential drugs or drug candidates in preventing and reversing the pathological changes. Such studies, which are proposed in this project, cannot be achieved without using 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 worked out protocols required in this project and estimated the numbers in each protocol based on published studies and test studies that we have performed so far. We will use the minimum number of animals required for our experiments whenever possible, and regularly consult a statistician for advice. We collect and review data from previous related work, from our own and from published work, to predict the expected results, and perform pilot experiments to allow the proper estimation of the number of animals required for each experiment. We used PREPARE (Planning Research and

Experimental Procedures on Animals: Recommendations for Excellence) to guide the design of the protocols.

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

We use online tools, such as the NC3R's Experimental Design Assistant (https://eda.nc3rs.org.uk/), to predict group sizes needed to detect differences with statistical significance based on the data collected during test experiments. Group sizes, gender, strain and age are matched for control and experimental groups. Sources of variability will be identified and minimised wherever possible. We will follow the PREPARE guidelines in our experimental designs.

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 perform most efficient breeding strategy for each GA colony following Home Office guidance and NC3Rs guidance: https://nc3rs.org.uk/3rs-resources/breeding-and-colony-management. We will optimise and standardise the protocols whenever possible. For example, variability in vascular phenotypes following gene activation with tamoxifen is minimised by ensuring the optimised tamoxifen dose is used. Data is collected by researchers blinded to treatment wherever possible and with experimental details recorded following the ARRIVE guidelines. We will always perform pilot studies for each new protocol to allow refinement of the experimental design. When we finish our experiments, we take extra tissues when feasible and store them and will share the tissues with other researchers 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.

We use mice that can produce certain human disease features to understand the components that cause and contribute to the disease. The genetically modified mice that we use generally carry ‘hidden’ mutations, such that almost all of animals are completely healthy until they are given the inducer to activate the mutation, reducing any clinical effects to the absolute minimum necessary for the project.

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

We cannot use nematodes or fruit flies to model these diseases because they don't have similar organs to the vertebrate and cannot provide the disease phenotypes observed in humans that we aim to investigate and treat.

Some of the diseases mostly manifest in adult human, hence we need to study these diseases in adult mice in addition to neonatal mice.

We study the disease progression over a period, from days to weeks, therefore cannot use terminally anaesthetised mice for this part of the work.

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

We will always use the minimum number of administrations where possible to achieve our scientific requirements in a study.

We will use the saphenous vein in the hind leg for blood sampling.

By using appropriate anaesthesia and pain-relieving drugs in the procedures to alleviate pain and discomfort, the protocols cause minimum possible discomfort to the animals. All Personal License holders (PIL) working under this Project License (PPL) will only work independently for any single procedure after their competency has been confirmed. Such information will be recorded and regularly checked using the online Mouse Colony Management System (MCMS).

Where animals are to be humanely killed for reasons unrelated to the scientific end of a procedure of the work described here, the Named Animal Care and Welfare Officer (NACWO) will be consulted to establish whether the animal tissues would be of value in other studies.

In terms of general mouse handling, we have moved to the tunnel method to reduce stress when removing a (post weaning) mouse from its cage.

From our previous experience, approximately 5 weeks following Tamoxifen injection to delete ENG gene in blood vessel cells, mice may develop enlarged hearts. A small proportion of the mice may also have bleeding in the lung. We have refined our protocol, performing daily monitoring after Tamoxifen injection, minimise the number of animals suffering and euthanise the animals when the clinical symptoms reach to the end points defined in the protocol.

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

We will perform all the experiments in the most refined way that we can do. We will discuss care and husbandry with Named Veterinary Surgeon (NVS), NACWO and Named Information Officer (NIO) whenever necessary, and use internal 3Rs Search Tool to access additional websites for information. We will refer to our internal updated Tamoxifen Guidance whenever possible.

We will use the ARRIVE guidelines for the reporting of our research: https://arriveguidelines.org/

If we later need to perform recovery aseptic surgery, we will follow LASA Guiding Principles for preparing for and Undertaking Aseptic Surgery (2nd Edition 2017) (https://www.lasa.co.uk/wp- content/uploads/2018/05/Aseptic-Surgery.pdf) for all surgical procedures.

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

We will regularly visit NC3R websites for updated information and subscribe to their newsletters. I am a member of NC3R Cardiovascular Network and will receive regular newsletter updates on 3R news and collaborative opportunities. We will use the experimental design tools on the NC3R website for each experiment. We will regularly discuss with the Named People at our facility to obtain any valuable resources for 3Rs advances.

32. Characterization of antibodies produced by cattle to rumen methanogens

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

Methane, Climate Change, Greenhouse gas, Global warming, Methanogen

Animal types	Life stages
Cattle	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 of this project is to generate initial, fundamental data, related to the immune response of cattle to methanogens which live in their stomachs and generate methane gas. The ultimate use of which would be to support evidence-based development of vaccines which may help reduce greenhouse gas emissions from cattle (and other ruminants).

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 many effects of climate change are well known, and a primary driver of climate change is the accumulation of greenhouses gasses in the atmosphere themselves a result of human activity.

Greenhouse gasses trap heat, and result in increased global temperatures. Livestock account for 14.5% of the global annual greenhouse gas emissions caused by human

activities. They impact the climate through various factors such as land use change, feed production, animal production, manure management, transportation, and release of methane - a natural byproduct of their digestive system. The work presented here will provide a proof-of-concept approach to support the development of a vaccine against the methanogen responsible for generating methane in the digestive system of cattle. This information will be used to help generate vaccines which could be used to reduce the production and release of greenhouse gasses from cattle and other ruminants, adding an additional tool for mitigating climate change while supporting food security and healthy protein production.

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

The outputs expected at the end of this project would be generation of fundamental data establishing if a vaccine approach could be implemented to reduce the growth of the microorganisms which generate methane in the digestive system of cattle, the methanogens. It is expected this new data would be published in peer reviewed journals and openly shared with the Global Methane Hub that coordinates all types of strategies to reduce the impact of livestock produced methane. This data would also be used to help create vaccines which themselves would aim to reduce methane production from cattle and other ruminants.

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

The fundamental research data generated from this project will help form part of a wider toolset which will underpin the rational creation and selection of a new generation of vaccines to help combat climate change.

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

This work is part of a larger collaborative project, whereby data generated will be shared and utilized by the partners. The expectation is that the data generated within this project will also be published.

Species and numbers of animals expected to be used

- Cattle: 28

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, and ruminants more broadly, have a specialised digestive system which enables them to digest long fibre foods such as grass, hay, and other plants. To investigate the immune response to the methanogen organisms living inside the digestive system, cattle provide an optimal platform both based on size, as well as the fact that the pre-existing optimized platforms for interrogating the immune response are already developed for cattle.

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

This project will examine the immune response of cattle to the methanogen microorganisms present in the digestive system of cattle, and as such, will involve inoculation of cattle with inactivated methanogen organisms by way of injection into the skin or muscle, and collection of blood samples to assess the responses in the blood. Saliva samples may also be taken by way of a non-invasive method.

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

Inoculation with inactivated methanogens and the collection of blood samples will require a needle to be introduced through the skin however no additional adverse effects other than the mild and transient effects of the needle penetration are expected. No side effects of inactivated methanogens to the cattle or their digestive systems are expected.

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 only mild severity during this study.

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?

Only whole animals, and in this case cattle as ruminants, can be used to assess the immune response to methanogens.

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

There are no non-animal alternatives which can be used in this project.

Why were they not suitable?

As stated, only animal models can be used to assess immune responses.

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 purpose of the pilot study is to characterize the humoral immune response to cattle methanogens, including identification of immune similarities between them. To this end, up to 5 different methanogens will be selected, which are the major methanogen species identified in the rumen. Prior studies analysing B-cell frequencies have suggested that 4 animals would be sufficient to generate the data required in this study, each group of 4 cattle being immunized with one of the 5 methanogen types. In addition, to screen for immune similarities, 2 groups of 4 cattle are planned to be sequentially immunized with different methanogens, based on the data generated from the initial studies.

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

These numbers are based on similar approaches in cattle using different immunogens and reflect the best evidence-based estimation of the minimum numbers of animals likely to be required for this study.

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

Continual assessment of animal numbers will be made based on the data generated given the humoral immune response to methanogens in cattle using the cutting-edge tools has not been undertaken before.

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.

Cattle, as a large animal ruminant model, will be used during his project. There is vast experience and expertise interrogating the humoral immune response of cattle.

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

The detailed antibody response of cattle to methanogens will be examined as the overall aim of this project, and therefore cattle are required, and terminal anesthesia / other methods of reducing sentience are not applicable.

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

Most importantly, ongoing review of approaches and results during the project will be undertaken and refinements applied / approaches refined to minimize the harms to the

animals.

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

Stimulating the humoral responses of cattle using antigens, and subsequent examination of these responses are activities which have been extensively studied at our establishment. The procedures undertaken in this project will be administration of substances and withdrawal of blood, and the LASA published guidance, and other referenced best practice guidance will be adhered to including "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes."

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

There are ongoing national and international links of our establishment with other research groups which enables the sharing of best practice in the fields of shared research. This, combined with extensive experience within the establishment and ongoing review of the scientific literature will assist in using the most refined methods and more widely applying the 3Rs where appropriate.

33. Management of genetically altered rodent lines

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Embryo, Sperm, In vitro fertilisation (IVF)

Animal types	Life stages
Mice	adult, embryo, 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?

To authorise the necessary procedures to generate or import new lines of genetically altered mice for use by scientists in a number of important research areas e.g., diabetes, cancer. It also covers the techniques to preserve these lines as frozen sperm or embryos (germplasm).

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 mouse is a very useful model system in which to study mammalian biology because of the similarities to humans, its small size, rapid reproductive cycle and extensively understood genetic background. This licence covers the necessary procedures to generate new lines of genetically altered mice for use by scientists in a number of important research areas, for example, the immune system, diabetes, cancer, and neurodegenerative disease. This ability is essential as many lines held elsewhere do not meet the FELASA SOPF exclusion criteria that is present in our high health status breeding unit and so the ability to rederive these lines is essential to the establishment as otherwise these scientifically important genetically altered lines could not be acquired and

they could pose a health risk to current animals within the unit. Therefore, this licence is requested to provide the legal authority to generate a 'clean' version of these lines to maintain the high level of health status to allow maintenance of the integrity of the science.

It also covers the techniques to preserve these lines as frozen sperm or embryos to allow lines to be preserved, "shut down" when not required and "tired", should there be a breakdown in health at the establishment, or should the scientist wish to share the mouse line with another establishment avoiding the transport of live animals.

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

Import and creation of new genetically altered mouse lines will support existing and new research programmes at our establishment, resulting in new information, publications and funding opportunities. Being able to freeze sperm and embryos means that we can avoid wasting animals by breeding them all the time. Instead, we can “store” them in frozen form so that they are only bred when needed. In addition, lines can be sent to collaborators as germplasm, avoiding the need for live animals to be transported, which can be stressful for them. We can keep stored germplasm for many years and this helps to protect against loss of lines due to a disease outbreak or issues with breeding performance, which is very important as almost all the lines are unique.

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

Researchers in local universities and their collaborators.

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

Close collaboration with other specialist centres, including the Mary Lyon Centre at MRC Harwell, the Jackson Laboratories and the world-wide community represented by the International Society for Transgenic Technologies (ISTT).

Species and numbers of animals expected to be used

- Mice: 7000

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 altered mouse lines are widely used in biomedical research. The activity of a particular gene may be disrupted (the so-called “knock-out mouse”) so that its contribution to normal health and the development of certain disease states can be studied in detail. Alternatively, very subtle change may be introduced, perhaps to make the gene product more similar to its human counterpart and thus improve the predictive value of the mouse to benefit human health.

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

We shall breed and maintain lines of genetically altered mice in which no deviation from

normal welfare is expected. We shall harvest embryos or sperm from these animals in order to have a frozen stock (and thereby avoid the need to continue to breed a line that is temporarily not needed). We shall send frozen embryos or sperm to other centres in order to distribute the lines we have and we shall import lines from other laboratories as embryos or sperm too, for “rederivation” into live mice here.

Animals are naturally mated and bred and kept in line with good practice, so breeding and maintenance of them is not expected to cause any significant adverse welfare effects.

To help make sure that animals don’t bring in potentially disease-causing organisms, and to avoid transporting live animals, we usually bring in new lines of mice using frozen germplasm. To get live mice from frozen material we inject hormones (similar to those used in woman undergoing the process of assisted fertility) into non-pregnant female mice. These hormones promote egg production. These mice are then humanely killed and their eggs are harvested, and fertilised in a test tube with thawed sperm from the mouse line we want to produce (in-vitro fertilisation (IVF)).

Embryos generated by this process are then implanted surgically into pseudo-pregnant females (i.e. mice whose physiological systems have been “fooled” by mating them with vasectomised male animals). This implantation, and the vasectomies, are surgical procedures conducted under general anaesthesia. The procedures are routine, and excellent rates of recovery are expected. All animals undergoing them will receive pain relief after surgery.

At the end of a procedure, animals may be humanely killed, some of them for the harvest of embryos or sperm for rederivation or frozen storage to preserve a unique line, or may be retained for further breeding under the authority of this licence or other project licences to which the mice might be transferred.

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

Those animals that undergo surgery will be expected to show some limited mobility for a few hours after recovery from the anaesthetic. They will be given pain relief to control any postoperative pain and monitored frequently until fully recovered.

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 great majority of animals are expected to experience "sub-threshold" or "mild" severities. Some, particularly those that have undergone a surgical procedure may experience "moderate" severity. No animal is expected to experience a "severe" event.

Breeding and Maintenance Mice: 95% sub-threshold; 5% mild Experimental Mice: 85% mild; 15% moderate

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?

Precise alteration of the activity of a specific gene in mammalian cells still often requires the production of a whole animal carrying the alteration of choice. So, even if most of the scientific effort is devoted to laboratory work on cell cultures, the mouse is still required as a source of the tissue or cells. Other genes express their activity in a number of tissues and organs and therefore can only be studied properly in the three- dimensional whole organism. This project supports researchers who require these live animals and cells.

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

Modern genetic alteration methods for direct intervention in cultured cells (e.g. CRISPR/Cas9). These are already in widespread use, so only those projects requiring live genetically altered animals need be considered here.

Why were they not suitable?

Alterations of cell cultures are rarely 100%, resulting in mixed responses to experimental interventions that can be difficult to interpret. Sometimes the relevant scientific "outputs" are in terms of the functioning of complex biological mechanisms, such as the immune and nervous systems, that cannot yet be recapitulated in 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?

The numbers of animals used will depend on the demand for the services provided within this licence authority. The most efficient breeding schemes will be used as well as following best practise guidance.

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

The minimum number of mice will be used to achieve success with cryopreservation and rederivation.

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 and superovulation 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.

Mice, as these are well-understood in genetic terms, have a rapid reproductive cycle and are regarded as good model systems for mammalian, and specifically human, normal health and disease states.

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

In many, but not all, regards, less sentient species such as flies and worms are not sufficiently similar to humans to generate translatable results. For example, their defensive mechanisms are very different from our immune systems, and they have much simpler nervous networks.

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

The major interventional procedure is the surgical re-implantation of embryos into female mice in order to carry them to the normal term and birth. This is a routine procedure and its centralisation in skilled hands ensures that the success rate is very high and that the animals receive the best possible post- surgical care. Non-surgical alternatives have been proposed but require the embryos to be grown to a certain stage in the lab to be successful. At the moment, this is very hard to do, but the NC3Rs have a project that is looking at how to make this easier and more successful. We shall continue to monitor the results of this and other projects that are working on non-surgical alternatives, with a view to adopting the methods they recommend wherever possible.

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

Home Office and NC3Rs guidance on best practice in breeding of Genetically Altered Animals and general breeding guidance from organisations with extensive experience in breeding mice (such as the commercial suppliers).

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

Close collaboration with other specialist centres in the field, subscription to NC3Rs newsletters and to professional journals in the field.

34. Porcine reproductive and respiratory virus studies of co-infection, immune responses, and evolution

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

Porcine reproductive and respiratory syndrome, Porcine circovirus, Immune responses, Co-infection, Experimental infection

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?

The project aims to improve understanding of the host-pathogen interaction of important viral infections of pigs. It will therefore examine the host responses of pigs and their impact on viral evolution as well as co-infections involving porcine reproductive and respiratory syndrome virus, porcine circovirus and hepatitis E virus, thereby also addressing the importance of such as occurring on farms.

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 an important problem for the pig industry in the UK and worldwide, leading to significant losses.

Many aspects of disease pathogenesis remain unresolved, and PRRSV infections seldom occur in isolation: additional pathogens are often involved. Investigation of co-infections with PRRSV and porcine circovirus type 2 (PCV-2) or hepatitis E virus (HEV) are required to foster our understanding of the way these co-infections happen in the field and how they influence each other. While some data is available for co-infections with PRRSV-2, little work has been carried out experimentally with PRRSV-1 which is notably different to PRRSV-2 and is the only PRRS-virus present in the UK. Therefore, these results will be more relevant to help inform disease control strategies here and in Europe where PRRSV-1 dominates.

The continually increasing diversity of PRRSV strains worldwide (related to their high mutation rate and their recombination potential) presents challenges both for diagnostic methods and for vaccine design. Existing vaccines are not very efficient nor efficacious to cover the diversity of PRRSV. More so, neutralising antibodies, widely regarded as crucial for protection, only occur late after infection and often after resolution of virus from blood and tissues. Hence other immune mechanisms seem to be more relevant for PRRSV protection.

Preliminary work has shown that the evolution of a PRRSV strain during infection of pigs can be characterized at a molecular level through full genome sequence analysis. However, studies of virus mutation in pigs at the level of different tissues will not only help to inform our understanding of population-level evolution of the viruses but also inform design of diagnostic tests and vaccines.

A better understanding of the dynamics of infection and co-infection with different PRRSV strains at the cellular level will help to assess the impact of recombination at the population level, informing appropriate control strategies for PRRS and consequently improved animal welfare and productivity in the pork industry.

Given the high prevalence and economic impact of PRRS in all major pig producing countries, this study has the potential to foster global economic performance in the pig production sector and specifically the economic competitiveness of UK farming.

Whilst the majority of PRRSV strains are not highly pathogenic, PRRSV infection suppresses the host immune system, allowing the establishment of secondary infections which results in more severe and chronic disease. Thus, improved PRRS control would have a significant overall animal welfare benefit.

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

At the end of the project work, we will have generated new information regarding the pathogenesis and protection from PRRSV and the evolution of PRRSV during replication in various tissues in pigs. We will have determined the likelihood of pigs showing variation in clinical signs from co-infection with PRRSV-1 and other pathogens versus single infections, and whether any differences are linked to the order and timing of infections. We will also have established how co-infection of PRRSV and additional pathogens affects the immunological responses in pigs, correlating this with clinical presentations and the potential for vaccine efficacy.

These findings will be communicated to interested parties including scientists, farmers, veterinarians and policy makers.

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

In the short term, the findings of these studies will benefit the scientific, veterinary and wider stakeholder communities, including diagnosticians, vaccine companies, farmers and livestock improvement companies, as well as policy makers working to improve the control of PRRS and porcine respiratory disease complex. In the longer term, improved disease control methods will aid better animal welfare standards and help secure sustainable food production globally.

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

The results of the studies undertaken under this project license will be disseminated to other researchers worldwide, in the form of peer-reviewed publications and of presentations at suitable scientific and veterinary meetings and conferences.

Remaining samples collected during the studies will be archived and made available for future research projects.

Species and numbers of animals expected to be used

- Pigs: 450

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.

Pigs are the only natural host of PRRS virus and therefore the only appropriate host for these studies of virus infection dynamics and immune responses. The project will focus on the respiratory form of disease, which usually occurs in juvenile to young adult animals. Study of the immune responses requires the use of juvenile to adult pigs, with a developed immune system.

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

Animals will be housed in groups on straw. Typically, pigs will be inoculated with a vaccine or vaccine candidate by injection into a muscle, under or into the skin or via an intranasal spray. Further booster immunisations may be applied. Up to 12 blood samples will be taken prior to and after immunisation to monitor immune responses to the vaccine. Some pigs will be euthanised typically no longer than 3 months after receiving the vaccines or vaccine candidates.

Other pigs, which may or may not have been previously vaccinated, will be inoculated with virus, by an intranasal spray, orally by suckling on a needleless syringe, or by injection either into a muscle or under the skin. Daily rectal temperatures and, blood samples, usually at 3- or more day intervals, but occasionally at 2-day intervals, will be taken to monitor disease progression. No more than 12 blood samples in total will be taken from an animal. Nasal and rectal swab samples will be taken at no more than 2-day intervals to monitor the potential for spread of virus. Animals will be euthanised typically no more than 3 weeks after inoculation with virus.

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

Blood sampling may result in minor swelling at the site of blood collection (neck) because of blood leakage (haematoma), which will resolve over a few days or weeks. There is a small risk of haemorrhage occurring due to a tear of the jugular vein. This adverse effect will be recognised by observing the animals closely following sampling for signs associated with haemorrhage, e.g. pallor, shaking, depression, shivering.

There will be stress associated with handling but this will be mild and transient and minimised by being performed by competent staff and pre-conditioning of the pigs. Very occasionally an animal may be injured as a result of an exaggerated response to being confined, handled or restrained.

Animals may develop clinical signs following PRRSV or PCV-2 infection for a few days duration. These may typically include reduced appetite, a lack of usual weight gain or slight weight loss, a mild fever, lethargy and weakness and a tendency to huddle together.

All the injection routes will cause momentary needle-stick pain. Collection of swabs will cause momentary discomfort.

Some pigs inoculated with novel vaccine formulations may experience minor swelling at the site of the inoculation, which will typically subside after 1 or 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)?

Clinical disease may be experienced in all animals inoculated with virus.
It is expected that most pigs (80%) will experience mild severity associated with sampling and early stages of virus infection. A proportion (20%) of pigs may experience moderate level severity for a short period following virus infection.

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 complexity of the immune system and the virus host interactions that impact replication within the host means that there are no alternatives that can replicate the vast array of factors that lead a vaccine to protect an animal from infection, to mimic the response to virulent virus, or to reproduce the genetic changes within the virus that occur during replication within animals.

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

Cell cultures, computer models.

Why were they not suitable?

Cell culture systems are available for PRRSV-1 and PCV-2, and will be used to produce the virus stocks used in these studies. These are not able to be used to answer the research questions regarding effects of co-infection, or virus evolution through different tissues in the animal. Similarly, immune responses to infection can presently only be evaluated in entire animals.

Computer models are used to analyse the genetic variability and evolution of the viruses, and while it is hoped that the data from these studies and others can inform the design of future computer models to predict virus evolution, these are not presently 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?

Estimated numbers have been determined using our previous experience of variation that occurs in the parameters we will measure in response to vaccination or virus infection.

The experimental plans for each individual study will be reviewed by peers to allow thorough examination of the experimental design. This will include review by expert statisticians to ensure the optimal number of animals are used to achieve the experimental aims. The design will be mainly based on the use resource equation.

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

We plan to test multiple experimental groups interrogating different vaccine formulations, examining immune responses to infection, and interactions between multiple pathogens, at the same time to minimise the number of control animals required. Small scale pilot experiments will establish the model for new virus infection models and for determining parameters such as the dose and formulation method of treatments before larger scale experiments. The experimental design assistant (EDA) from NC3Rs will be used for specific study designs, and a biostatistician will be consulted on the appropriate sample size as part of any study design.

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 variation standard protocols will be applied, for example for preparation of test treatments and challenge virus stocks, and for performing the inoculations and laboratory

analysis. To control variability and allow smaller group sizes animals will be of similar genetic background, age and weight, with a good health status. Tissues from the project will be archived for our own further investigations and where possible 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.

The project involves infection of pigs with PRRS virus 1, PCV-2 virus, or co-infection with two viruses. Vaccination of pigs followed by infection with virus will also be carried out.

The infections will be carried out using virus strains that have been previously characterised, and for which established clinical scoring systems are available for clinical monitoring and endpoint assessments. If infection with a new strain is required, a pilot experiment in a small number of animals will be used to characterise the clinical signs and inform the optimisation of clinical monitoring and scoring systems before larger studies are carried out. Definition of humane scientific end points appropriate to the objectives of each study are used to ensure that animals are euthanised at such a point that they experience the least pain, suffering, distress or lasting harm necessary to meet the scientific objectives. However, to evaluate the effects of co-infection, immune responses to infection, or protection from disease by vaccination or treatment, it is unavoidable that some animals will experience discomfort associated with the early stages of disease.

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

Pigs are the only natural host for the viruses to be used in this project, with no other animal species producing the same responses to infection with virus or vaccination.

To study the immune response, it is necessary for animals to be immunocompetent with a developed immune system and so immature stages cannot be used. It is not possible to achieve the scientific aim of characterising multiple infections caused by different viruses in pigs using other species.

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

Our clinical monitoring scheme includes actions to increase monitoring of animals exhibiting clinical signs. Pre-start meetings are held before each study, and wash-up meeting after each study to include personnel carrying out procedures, caring for and monitoring animals, NVS, NACWO and scientific staff. These meeting will identify suggestions for refinements, e.g. reviewing the clinical scoring system to refine end-points, which will then be implemented as applicable.

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

We will use the Norecopa PREPARE guidelines and information from associated web sites, such as specific guidance for the use of farm animals in research.

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

Information from the NC3Rs web site and newsletters will be regularly reviewed. Advances in methods relating to the 3Rs, from colleagues in the establishment or from relevant external colleagues or publications which are regularly disseminated across the establishment will also be regularly reviewed.

We will be in regular contact with other researchers worldwide carrying out work in this area, and new information on procedures for similar studies will be evaluated. All relevant advances will be incorporated into study designs and procedures wherever applicable.

35. Regulation of hypothalamus and pituitary functions by Glucocorticoids

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Stress response, Glucocorticoids, Zebrafish, Hypothalamus, Imaging

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 how stress hormone, Glucocorticoid, changes brain cell activity and 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?

Glucocorticoid (called cortisol in humans) plays a major role in limiting stress after it has been initiated, but how it regulates brain cells to limit stress response is unclear.

We use zebrafish as a model to reveal the underlying mechanism behind Glucocorticoid inhibition of stress response. This knowledge is important to identify therapeutic targets for stress-induced disorders.

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

Outputs will be new knowledge that will be disseminated in the form of publications.
Who or what will benefit from these outputs, and how?

The successful completion of this project will have a major impact on academic community studying stress. It will also generate information with a huge translational impact. The molecules identified here can be promising therapeutic targets for controlling adverse stress reactions in humans.

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

We will disseminate new knowledge obtained here using all available channels including formal scientific publications, presentations in scientific conferences and workshops, and scientific social media channels. In addition, we will utilize knowledge dissemination channels to reach general public including University press office, scientific outreach program such as Pint for Science, etc.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): Adult zebrafish - 8000, Larval zebrafish 5-12 day old- 7880

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 stress response is complex and require experiments in intact animal. So far most of stress research has utilized rodent models. However, our work has established that zebrafish is a good alternative model to rodents for stress research as the stress response system is highly similar in different vertebrate species. We use mostly larval stage where stress network is already established in zebrafish but perform some experiments in adults as well.

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

We create genetically altered animals that allow us to record activities of cells using microscopy. We then ask how brain cell activities change when cortisol is applied. We use various techniques to identify the molecules that help cortisol to carry out its action. The roles of these molecules are tested by creating genetically modified animals that carry specific changes in these molecules. We then analyse these animals using microscopy and behavioural tests.

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

All the procedures that we carry out are of mild severity. We video record freely moving natural animal behaviour. Further we record their behaviour after they are exposed to acute stressor which are very short in duration (max 10 min) and the treated animals recover fully afterwards. While the animal's behaviour is affected while acute stress is being applied there is no lasting adverse effects. We do not expect weight loss, tumour development, nor lasting pain. The most invasive procedure that we perform is recording cellular activities and imaging under microscopes. However, we use fully anaesthetized animals for these procedures and therefore we do not expect them to experience pain

during 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)?

8.9% non-recovery, 91.1% 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?

How hormones regulate brain cell activity and function cannot be studied using computer models or isolated cells alone. This question requires an intact animal.

Traditionally the question that we are posing here was addressed using rodents, but zebrafish offers most refined alternative animal model.

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

We have considered in vitro cell system.

Why were they not suitable?

The specific cells that we are proposing to analyse here form a tightly regulated structure in animals. This is difficult to study fully without using an intact animal.

Isolated cells cannot mimic the complexity of structures that are found inside of 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?

Most of the experimental methods to be used in this project have already been tested extensively in zebrafish by our group and others. This means that we can estimate with a

good confidence the numbers of animals needed to achieve the experimental goal. Wherever possible we used mathematical calculations to determine the number of animals needed for minimum detectable effect.

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

We use reference online tools such as the NC3R's Experimental Design Assistant. To reduce experimental variability, all experiments are conducted using standardised established protocols in controlled setting. There are no experiments proposed here that utilise previously untested protocols. In cases where we need to establish exact dosage regime, we use younger embryos at unprotected stages to carry out 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?

We will collaborate with researchers in the department of mathematics and statistics at the University of Exeter. The data that we generate will be analysed by them to develop a rigorous mathematical model. The mathematical modelling will be carried out similar to that reported in Walker JJ and Lightman S in 2010. In this work ordinary differential equations (ODEs) based approach was used to characterize cortisol fluctuation. Using numerical simulations and continuation methods, a range of cortisol values can be approximated under different experimental condition. This model will guide our experimental design and hypothesis. This approach will help us to improve our effect size and variance leading to smaller numbers of animals that we use for this project. Further whenever possible, we will freeze the brain and body samples of the experimental animal tested in behaviour or imaging for further post-mortem 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.

Zebrafish is the most refined animal to study the effect of stress hormones on neural circuits, physiology, and behaviour. This question has been traditionally studied in rodent models, especially using mouse. The zebrafish offers an excellent alternative as many of the questions related to stress hormones can be studied using larval zebrafish at stages when they are not considered to be protected animal.

Importantly key functions of the stress hormones is similar throughout vertebrates. Therefore, many questions regarding their effects and regulation can be studied in zebrafish and do not require mammalian models.

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

As the stress hormones studied in this project is a vertebrate feature, the questions addressed here cannot be carried out using invertebrate genetic models such as fruit flies or worms. Studying animal behaviour is not possible using cell lines.

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

All animals are carefully monitored on daily basis by trained staff. In addition, individual researchers on this project (trained postdocs or PhD students) will monitor the experimental animals that they are using carefully. Animals will be monitored for adverse effects such as changes in weight, changes in mobility, lumps, eye colour, gills, and abnormal respiration. If these are observed animals will be treated accordingly, and animals that develop symptoms that impact the welfare of the individual 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 PREPARE, ARRIVE, SAMPL, and TOP guidelines to assist with planning animal research. We will use additional resources available from NC3Rs and Laboratory Animal Science Association.

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

We will regularly check information on NC3Rs website, we’ve signed up to the NC3Rs newsletter, and attend Regional 3Rs symposia. We will stay up to date with 3Rs information with discussion with the Named Information Officer/ Head of Biological Services and her team (NACWOS/ NVS and HOLC).

36. The role of host-gut microbiota-diet interactions in modulating cardiometabolic risk factors and colonic 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

Gut microbiota, Metabolites, Colon cancer, Nutrition, Cardiometabolic health

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 seeks to understand how the chemicals produced when our bodies interact with the food we eat and the tiny organisms in our guts can affect our risk of developing colon cancer and impact our cardiometabolic health. The cardiometabolic health is related to a group of factors, such as inflammation, fat concentrations in our blood, body fat distribution and problems related to blood sugar regulation. These factors can impact the likelihood of developing diseases, such as heart disease 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?

Diseases like cancer, high blood pressure and diabetes are big health problems worldwide. For example, colon cancer is the second leading cause of cancer-related deaths worldwide. In 2020, more than 1.9 million new cases of colorectal cancer and more than 930,000 deaths due to colorectal cancer were estimated to have occurred worldwide.

Approximately 42,900 new bowel cancer cases in the UK every year. It affects both men and women. Additionally, more than 50% of UK population have at least one of abnormal factors (e.g. inflammation, fat concentrations in our blood, body fat distribution and problems related to blood sugar regulation), and this rate has been shown to increase over decades.

To prevent these diseases and reduce their impact on society, we need effective strategies. Research shows that how our bodies, the food we eat, and the tiny organisms in our guts interact can affect our risk of these diseases. But we're not sure exactly how this interaction directly affects our risk, and we don't fully understand the reasons behind it.

Figuring out this cause-and-effect relationship can help us develop better ways to prevent these diseases.

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

Our goal is to collect information to better understand how the way our bodies, gut microbes, and diet interact affects our gut and overall health. We plan to share this information by publishing it in freely accessible scientific journals and making the data available through public data repositories.

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

Researchers in this field will gain immediate benefits from our project because they'll have access to our publications and experimental data while the project is ongoing. In the next 3 to 8 years, we hope to test our findings in people by making changes to their diets and gut bacteria to see how it affects their gut and cardiometabolic health. In the long run, all of this research could help us develop strategies to reduce risks of developing colonic cancer and other diseases, such as obesity, diabetes and heart disease.

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

To make the most of our research, we will share our work through presentations at conferences and webinars, publish our findings, methods, and data for others to use, and engage with the public to spread the results. We will share unsuccessful results so that the scientific community is aware not to repeat the work or able to improve their methodologies. We also plan to strengthen our partnerships and collaborate with dieticians and clinicians to make sure our research can be applied in real-life healthcare settings.

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.

Researchers often use mice to study how what we eat and the bacteria in our guts affect our health. Compared to other animals like zebra fish or fruit flies, the structure of the bowel of mice are more similar to ours. We can change the types of bacteria in their guts and what they eat to see how it affects their health, specifically cardiometabolic risk factors, which can lead to increased risk of developing metabolic diseases, such as obesity and diabetes. Mouse models help us understand how our bodies, gut bacteria, and diet all work together.

Mice are also used to study a type of colon cancer that's similar to what happens in humans. We have special mice that get this cancer, which helps us learn how the way our bodies, gut bacteria, and diet interact can lead to colon cancer. We use adult mice for this because they start getting these tumours when they're around 11-18 weeks old, which is when we want to study them.

To see how our bodies, gut bacteria, and diet affect our gut and overall health over a longer time (like 4-6 months), we'll use adult mice that don't have cancer. This way, we can observe what happens when we change their diet and gut bacteria for a few months.

Mice share a significant amount of genetic and functional similarity with humans, making them a valuable model for understanding how the maternal gut bacteria and diet affect the offspring's health. This model will in addition allow us to assess the combined effects of the maternal gut bacteria and diet and offspring's diet on the health of the offspring. We will use adult female mice for breeding and their offspring, both male and female, will be studied after weaning at 3 weeks old up to mature adult.

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

Mice will have one of the following three typical scenarios based on the specific objectives of the experiments:

To study how the gut microbiota and diet impact the colonic cancer

This experiment is typically up to 10 weeks using genetically altered mice that develop intestinal tumours typically from age of 11 weeks old. We will start the experiment when the mice are 4 weeks old. They will be housed in groups and given about a week to get used to their surroundings and to have a stable starting point for their metabolism and gut bacteria. We'll take blood samples at the beginning to establish this baseline. Next, we're going to tweak their diet or add some supplements for about 10 weeks. This might make them gain a little weight. While they're on this special diet, we're going to give them antibiotics in their water for a week and feed them bacteria through a tube 5-7 times, typically once per week, to maintain the transplanted bacteria in their guts. We might see small changes in their body weight because of these gut bacteria changes. We'll take further blood samples in the middle and at the end of the 10 weeks. We'll image the mice once to see if there's any change in tumour growth. After that, we'll use special medicine to make the mice to sleep and keep them from feeling anything, and take a final blood sample from their hearts. We'll collect tissue samples from their gut, liver, brain, heart, kidney, spleen, fat, and tumours. These samples will be used for different types of analysis like studying metabolism, gene function, tissue structure, and immune cell activity. All of this will help us figure out how different diets and gut bacteria affect tumour development.

To study how the interactions of gut microbiota and diet modulate the

cardiometabolic risk factors

This experiment is typically up to 12 weeks. We will study mice, both normal ones and ones without the Apoe gene, when they're 5 weeks old and housed in groups. Mice without Apoe gene are prone to developing abnormal lipid levels in the blood and heart diseases. We want them to get used to their environment for a week to have a stable starting point for their metabolism and gut bacteria. We'll take blood samples at the beginning. Then, for 11 weeks, we're going to change their diet, give them extra nutrients, or limit their calories. We'll also give them antibiotics in their water for a week and feed them bacteria through a tube in their mouths 5-7 times to introduce the specific bacteria we're interested in studying. Throughout the experiment, we'll measure two times how well the mice process sugar and respond to insulin. These tests will be done by taking a drop of blood from the tail at a series of time points following an oral dose of glucose or an injection of insulin. We'll image them to see how much body fat they have and where they are across the body. We'll take more blood samples every 3-4 weeks. When we finish the experiment, we'll use special medicine to make the mice to sleep and keep them from feeling anything, and take a final blood sample from their hearts. We'll analyse this sample to see what kind of fats, proteins, and markers of inflammation are in their blood. We'll also collect tissue samples from their gut, liver, brain, heart, kidney, spleen, and fat. These samples will be used for different types of analysis like studying metabolism, gene function, tissue structure, and immune cell activity. All of this will help us figure out how different diets and gut bacteria influence the health of the gut and other organs.

To study how the maternal gut microbiota and diet impact the offspring's metabolic health

First, we work with female mice that aren't pregnant and are usually around 6 weeks old. They live together in groups for a week to make sure we start with a stable baseline for their metabolism and gut bacteria. We take blood samples at the beginning. We then change their gut bacteria, usually by changing their diet, for about 4 weeks to stabilise the changes. We use metabolic cages to collect their urine and poop over a whole day, and they keep living in groups after that. We test how well they process sugar once. We also take another blood sample before they are paried with male for breeding.
After pairing, we check for signs of pregnancy. We do a blood test and a sugar test during pregnancy, and measure their body weight. We will not change their diet during pregnancy. We don't do imaging and use metabolic cages on pregnant mice.

Once the pups are born, we weigh them every 2 days until they're 3 weeks old. The female mice will be humanely killed after their pups are weaned.

We will study the offspring until they're about 19 weeks old. After the weaning, we take one more blood sample and do one more imaging session to see how the fat distribute in their bodies. As they grow up, we take blood samples every 3-4 weeks and test how well they process sugar and insulin. We also check their body fat distribution again. The offspring will not be bred.

Finally, the animal will be humanely killed. We collect their blood from their hearts and measure fats, proteins, and markers of inflammation. We also take tissue samples from their gut, liver, brain, heart, kidney, spleen, and fat. We study these samples to understand how different diets and gut bacteria affect the health of their organs.

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

Tumours:
The genetically altered mice used in the experiments are expected to develop tumours at around 16(+/-3) weeks of age in male or 18(+/-3) weeks of age in female.

Tumours are usually present in the bowel for 2 weeks. If the animals show signs like having blood in their poop during this time, they will be humanely killed.

Another genetically altered mouse model for intestinal tumours will show the following clinical signs: anaemia (seen as pallor of mucous membranes, lethargy), hunched posture, abnormal gait, inactivity or reduced appetite, progressive adenoma from 11 weeks of age until reaching multiple adenomas at age of around 18 weeks.

Tumours are usually present in the bowel for 2 weeks. If the animals show signs like having blood in their poop during this time, they will be humanely killed.

Metabolic cages:
Initially majority of animals might be agitated (e.g. decreased water and food intake, making excessive noise) when placed in the metabolic cages. Mice in metabolic cages will be monitored twice daily and will be removed if they show agitation for 1 hour or longer. Mice may lose weight in the metabolic cages. Mice will be humanely killed if a 15% reduction in weight measured one day after the use of metabolic cages, compared to the body weight measured prior to introducing the mice to the metabolic cages.

Dietary manipulation:
If animals are fed with western diet, weight gain, oily hair coat, over-grooming, itching and irritation of skin due to oily hair coat, increased drinking and urination due to weight gain, and likely dermatitis (itchy and inflamed skin).

Caloric restriction:
Animals are expected to lose weight due to caloric restriction and this is a part of expected results. Mice, who are calorie-restricted for more than 10 weeks, may have excessive weight loss, defined as 15% weight loss compared to the animal’s maximum adult weight.

Pregnant mice are expected to have a lower body weight gain and a lower birth weight of the offspring, compared to non-caloric restricted group.

Gut microbiome manipulation:
<10% of mice may experience diarrhoea and changes in body weight as a consequence of the altered gut microbiota. Mice with diarrhoea will be humanely killed.

Single housing
It may cause distress to the animals. This step will be avoided where possible. typical length is 10 weeks with a maximum of 5 months. Mice which show marked behaviour changes such as stereotypic behaviour will be humanely killed.

Imaging
it possibly causes dehydration. Supportive fluids will be provided immediately to avoid animal suffer. Glucose tolerance test:
High blood sugar (<2% of incidence) for less than 2 hours and blood loss from the incision (<5% of incidence). For pregnant mice, it may cause distress and less weight gain. Mice will be humanely killed if no weight gain 1 day after the procedure compared to baseline weight at the start of the procedure.

Insulin tolerance test:
Low blood sugar, which happens very rarely (less than 2% of the time), can cause symptoms like shaking, increased anxiety or indifference, tingling sensations, fainting, and, in rare cases, seizures. For pregnant mice, it may cause distress and less weight gain. Mice will be humanely killed if no weight gain after the procedure compared to baseline weight at the start of the procedure.

Blood sampling from pregnant mice
It induces stress. Mice will be handled and constrained by cupping during the procedure to minimise the stress. Maximum one sampling during pregnancy.

Breeding
Mice may have difficult or abnormal labour, vaginal prolapse, or signs of pain or distress (hunched posture, or abnormal grooming 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)?

51.6% sub-threshold; 21.3% mild; 27.1% 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?

This project aims to study how the chemicals produced when our bodies interact with the food we eat and the microorganisms in our guts affect colon cancer and our overall health. Right now, we don't have a good way to recreate this interaction in a lab dish.

While we can study the effects of nutrition and gut bacteria in some people, it wouldn't be ethical to do so for this project, especially when looking at how it might affect not only one person but also their future generations.

Also, the chemicals we want to study inside of the bowel is different from what you find in poop. It's hard to get the samples we need from healthy people without doing invasive procedures. So, we're using animals to help us learn more about how all these things work together. This will hopefully lead to better ways to prevent colon cancer and metabolic diseases.

Which non-animal alternatives did you consider for use in this project?
Chemostat system, co-culture of mammalian cells and bacterial cells, computational modelling and simulations, and human studies.

Why were they not suitable?

The chemostat system we use helps us grow gut bacteria with different food components. But it can't mimic the way our bodies, gut bacteria, and diet all interact because it doesn't have the parts our bodies use, like digestive enzymes, bile acids, and nutrient absorption.

Another method, where we put mammalian and bacterial cells together, can't capture how different microbes in a community interact, which is really important for understanding how our gut bacteria work.

We've also tried using computer models and simulations, but the knowledge in this field isn't advanced enough to make really good models, and these models can't show a clear cause-and-effect link between all these complex interactions and disease risks.

The best way to study this would be in human volunteers, but we can't do that for some of the specific things we want to measure, like how much tumours grow or how healthy the offspring of the volunteers will be in the long run, because these effects come from the way our bodies, gut bacteria, and diet interact over a long time.
We will use alternative approaches where suitable and describe. For example, we have established bacterial and mammalian cell culture experiments to explore the metabolic activities of the gut bacteria and the impact of metabolites of interests on the phenotypes of the mammalian cells. Parallel in vitro work will be carried out to support the in vivo work and these include bacterial culture with different nutrient substrates and culture of mammalian epithelial and immune cells treated with various microbial and dietary metabolites. We will seek opportunities of using gut-on-a-chip device, for example, the Emulate human colon intestine-chip system.*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?

We have carried out such research before, breeding both mice with cancer and without it. To estimate how many mice we need, we considered factors, such as how many baby mice are born in a litter, what kind of mice they are, how challenging it is to breed them, and how often we need to do it. We also worked with a statistician who used data from our previous experiments to figure out the minimum number of mice we should use to make sure our results are reliable. Typically, these calculations show that we need 18 mice per group to achieve the quality of the results we are aiming for.

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

We employed the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental design, practical steps and statistical analysis utilising the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods. 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?

We have established standard operating procedures (SOPs) and shared them within the research team to reduce experimental variability induced by techniques and researchers. New team members will be trained by experienced members prior to the start and will gain confidence and independence gradually. We will conduct pilot studies to determine the concentrations of dietary supplementation or the dose of gut bacteria given to the animals. Breeding will be set up based on the experimental design and the required number of animals. We will harvest as many tissues as possible at post-mortem in case some of them become relevant/interesting for further exploration. We will be willing to share tissues with other groups, for example, we have collected the mouse tails from our project for another research group to test their parasite diagnosis device.

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 Apc mutant mouse is used to study strategies for preventing colon cancer.

These mice develop multiple tumours, mainly in the small intestine. We maintain this strain by breeding males with a mutant Apc gene with C57BL/6J females because female mice with Apc gene mutations can experience anaemia (a condition that the body doesn't have enough of the red blood cells that carries oxygen) and intestinal tumours, which can interfere with pregnancy. We avoid breeding males once tumours develop. For the FabplCre;$Apc^{(15lox/+)}$ mice, which develop tumours in the large intestines like humans with familial adenomatous polyposis (FAP), our focus is on early symptoms, and we minimise animal suffering. In non-cancer models, we use wild-type animals.

To study the effects of diet and gut bacteria, we manipulate the gut environment by changing the diet, providing dietary supplements, and altering the gut microbiota. We explore options like giving antibiotics in drinking water and providing dietary supplements in drinking water or food pellets instead of oral gavage.

We also use caloric restriction (up to 50% reduction, typically 20%) to mimic people who have had weight loss surgery. To ensure animal well-being, we set a humane endpoint at 15% or more weight loss.

Published studies have shown that animals housed in the same cage had similar microbial composition compared with animals housed in different cages, so called ‘cage effects’. Co-housing, where the microbiota can be easily transferred between animals, can compromise the results and introduce bias into the study. Some animals will be single-

housed to avoid "cage effects", and we provide enrichment like aspen balls. Metabolic cages are used to collect urine and feces, and we introduce animals to these cages gradually to reduce distress.

Metabolic cages will be used to collect 24-hour urine and faeces in certain experiments/time points. To reduce their distress, we will gradually introduce them to metabolic cages by increasing the time spent there. Other options, such as use of sand that does not mix well with water, will be explored during the project.

Oral glucose and insulin tolerance tests are conducted to assess glucose metabolism. Typically, we measure the changes in blood glucose levels over a 1- to 2-hour interval following a dose of glucose or insulin. The published literature suggested that fasting 6 hours in mice prior to these tests are enough to assess insulin action within a more physiological context. The minimum recovery time between repeated sampling is one week.

We will collect blood samples using a method called venepuncture. The amount of blood we take will depend on how many times we need to measure, the animal's weight, and how often we collect samples. Normally, we take blood every two weeks, but sometimes we may need extra samples for scientific reasons. We'll always make sure the total amount of blood we take stays within the recommended guidelines to keep the animals safe. We'll use sterile techniques to prevent infections in the animals.

Imaging will be used to assess the body fat distribution or tumour size. Body weight is not the best readout for obesity since the fat distribution is far more important than the overall body weight with regards to one’s disease risk. Imaging techniques such as ultrasound, magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT)/positron emission tomography (PET)/ computerised tomography (CT) or optical will be used to check the tumour size and numbers, and one imaging method on any one day. Animals will be monitored regularly and a score sheet for assessing animal health will be applied. Animals will be offered soft and energy-dense food when necessary to ensure animal health.

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

Fruit flies and nematodes have been used to explore how the gut microbiota works. However, these models have limitations. They have simpler gut structures, less complex gut microbe communities, and different eating habits compared to rodents and humans. So, they're not a good fit for this project.

The interactions between the gut microbiota and their host, as well as the impact of diet, are different in younger creatures compared to adults. Since our project aims to recreate the complex interactions that happen in adult humans, it makes more sense to use adult rodents.

Furthermore, we want to study how these interactions affect not just the adults but also their offspring in the long term. So, we'll be monitoring the health of both mothers and their babies from birth.
Terminally anaesthetised animals will not be suitable since we aim to investigate the health consequences of dietary/gut microbial manipulations.

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

We will increase monitoring when a procedure(s) is carried out or animals are expected to start developing tumours. Anaesthetics will be used if animals are to undergo a procedure which might be uncomfortable or painful and pain relief administered as it would be in humans. Liquid food is easier to eat and digest and will be fed when necessary. Animals will be introduced to a different type of caging if needed for the procedure, by gradually increasing the time spent there until they are familiar with it. All animals will be housed in groups where possible, with nesting material and play tunnels, and fed according to current institutional 'best practice'.

We will include acclimatisation to handling for mice that are going to be handled as pregnant dams. The most refined method of administration of substances will be used (e.g. oral in water vs oral gavage), and the use of hydrophobic sand over metabolic caging will be explored.

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

Resources are available including guidance and publications from the NC3Rs and Laboratory Animal Science Association, the ARRIVE and the PREPARE guidelines, as well as "Refining procedures for the administration of substances" for administration of substances.

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

We will stay informed through multiple channels including X (@NC3Rs) and 3-minute 3Rs Podcast. We will meet our NC3Rs Programme Manager and attend local and regional 3Rs symposia.

37. Understanding renal and intestinal phosphate and sodium transport 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

Phosphate, Sodium, Diabetes, Chronic Kidney Disease, Transport

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 kidneys play a critical role in maintaining ion levels in our body, which if impaired can have significant effects on our health. The project aims to focus on two ions, phosphate, and sodium, and to investigate whether there are novel ways in which we can prevent levels of these ions from becoming too high in our blood and damaging the cardiovascular system in people with kidney 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?

Kidney disease is strongly linked to the development of cardiovascular disease. It is widely recognised that eating too much salt has a major impact on our health, but the impact of dietary phosphate, which is found in high levels in processed foods, can also be detrimental, especially in people with reduced kidney function. The study will increase our understanding of the processes involved in dietary phosphate absorption and the impact

that this can have on heart health. This knowledge is necessary for developing drugs to target intestinal phosphate transport for disease treatment and prevention.

Identifying novel ways in which the kidney controls blood pressure via sodium handling also has the potential for developing new antihypertensive drugs.

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

The study will provide new information about the basic physiology of dietary phosphate absorption and the impact that this can have on heart and bone health. The project will also determine if diseases such as kidney failure or diabetes alter these processes and further increase the risk to our health. It will also increase our knowledge of the role of the kidney in controlling blood pressure and the impact that salt in our diet has on this process. 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?

While it may not have an immediate social and economic benefit, the information generated will be of value to academics, clinicians and nutritionists interested in promoting healthy eating, both for lifelong health and for improvement of chronic disease. This scientific knowledge is also necessary to allow the long-term development of drugs to target intestinal phosphate transport and renal salt transport for disease treatment and prevention.

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

Presentations to the academic and lay communities and publications in open-access journals will allow new information to be shared and used by others. Negative results will be published whenever possible and complex data sets will be shared with others using online data repositories.

Species and numbers of animals expected to be used

- Mice: 1000
- Rats: 1400

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 key aspect of the work is to establish how the whole body responds to changes in dietary phosphate intake and what effect this has on the heart. It also aims to see whether diseases such as diabetes and kidney failure change these responses. While cell culture experiments can be used to investigate how an individual cell responds to a specific treatment, they cannot provide information on how the whole body responds. Therefore, animal models are required, and in most instances, the rat is the animal of choice. Surgical or chemical induction of kidney disease or diabetes is generally easier and more reproducible in rats, and metabolic cage studies are less stressful for rats than mice. There are also known differences between the processes for intestinal phosphate absorption in

rats and mice, with growing evidence suggesting that the rat more closely mirrors the processes that occur in humans.

When studying how phosphate is absorbed from the diet and if this process can be inhibited, both juvenile and adult animals will be used. This is because the processes may be different in juvenile animals since phosphate is needed in high amounts for bone development. In contrast, adult animals will be used when investigating the impact of disease on phosphate homeostasis, as the diseases being studied are more common in adult humans. For some experiments, knockout (KO) mice for proteins involved in phosphate transport are needed. Their use will allow the contribution or adaption of each transport pathway to be confirmed in response to the different treatments or drugs that are being investigated.

For the studies investigating the role of the kidney in blood pressure control and the impact of dietary salt intake on this process, typically adult mice will be used. This is because we have access to knockout mice for genes that we think are involved in this process.

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

Models of disease may be induced in rats or mice. This includes feeding of diets high in fat that cause obesity (10-week treatment) or diets that contain a drug (adenine) which causes kidney disease (max16-week treatment). Alternatively, animals may have chemically-induced diabetes or surgically- induced chronic disease, with animals kept for a maximum of 6 weeks after induction of the disease. Animals with genetic mutations that cause obesity or diabetes, or that change the process of intestinal phosphate transport will also be used.

Normal animals or animals with one of the described diseases will typically be given test substances by injection using standard routes (intravenous, intraperitoneal, intragastric) for short-term treatment (max 5 days) or in their drinking water for long-term treatment (max 5 weeks). They may be given diets that contain high or low amounts of phosphate or salt, or that contain specific phosphate preservatives (max 5 weeks). They will receive a max of 2 manipulations per animal. Animals may be placed in metabolic cages to test the impact of these treatments on phosphate homeostasis. For rats this will be for a maximum of 24 hours, while for mice it will be for a maximum of 6 days.

The final procedures will be undertaken under non-recovery anaesthesia and will be used to establish the effect of the different treatments on transport of phosphate or salt by the intestine and kidney. It will also allow for collection of blood and different organs for further analysis.

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

The surgical procedures that are used to cause kidney disease will be done under general anaesthetic. These animals will experience some discomfort after surgery and some mild to moderate pain. For all surgical procedures the possibility of infection is minimised by using the same clean conditions used for human surgery, with the animals receive painkillers and post-operative care just like people recovering in hospital. While the animals will show clinical signs of kidney failure, they display no significant adverse effect at the endpoints proposed in this study. The same applies for animals that have genetic or diet-induced obesity, diabetes, or kidney disease.

Based on our previous studies, animals undergoing any of the dietary phosphate or salt changes, oral treatments or injections display no long-term adverse effects. Some animals may experience diarrhoea but recover within 48 hours. Metabolic cage experiments are known to lead to stress and weight loss, particularly in mice. Unfortunately, to obtain good results from these experiments mice need to acclimatise to their environment before samples can be collected. However, to minimise stress, the length of time animals are confined in the metabolic cages will be kept to a minimum and enrichment in the form of a nest box or crawl ball will be included in the cage. Body weights of the animals will be monitored as a guide to general health. If an animal has weight loss approaching 20% of their starting weight, it will be killed by a Schedule 1 Method. If any of the procedures result in or induce evidence of suffering in an animal, or in any way affects normal behaviour, the animal will be humanely killed using a schedule 1 method unless, in the opinion of a veterinary surgeon, such complications can be treated promptly and successfully.

The final procedures will be undertaken under non-recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and may experience mild distress and no 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)?

This licence contains multiple protocols that may generate either mild or moderate severity. For example, protocols where the aim is to investigate the impact of kidney disease or diabetes, we expect the animals to experience moderate severity, while sham animals would only experience mild and transient discomfort from wounds after surgery. For each species the following percentage of severity has been calculated.

Mice: 78% mild, 22% moderate

Rats: 60% mild, 40% moderate

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?

An important aspect of the proposed work is to establish how different organs and hormonal pathways responds to changes in dietary phosphate or salt intake, what effect this has on the heart and bones or blood pressure, respectively. We also aim to establish whether diseases such as diabetes and kidney failure change these responses. This whole-body approach makes the use of animal research critical.

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

My research uses in vitro and in vivo approaches to investigate the cellular and homeostasis mechanisms involved in epithelial transport. A significant proportion of the work involves the use of cell culture experiments to investigate fundamental mechanisms at the cellular level.

Why were they not suitable?

While cell culture is good for establishing mechanisms in individual cells, it is unable to determine how different organs and hormonal pathways interact to regulate nutrient homeostasis. It is also unable to establish if interventions aimed at reducing intestinal phosphate transport or stabilising phosphate homeostasis have positive effects on renal and cardiovascular outcomes in the setting of CKD.

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 number of people working within this licence and previous experience of animal numbers required for a project of this scale.

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 most of our experiments, data from previous, similar experiments, by our laboratory and published by others, can be used to establish the dose and treatment duration meaning that pilot studies are not typically required. The results from our own previous studies are also 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 with 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?

Under the guidance of the experienced staff in the comparative biology unit we will strive to achieve the most efficient breeding of genetically altered animals. We also strive to maximise the data generated by each animal, i.e. changes in renal and intestinal transport function and transporter levels will be established in one animal. This approach not only reduces animal usage but also allows more detailed and precise interpretation of data. In addition, we collect and store different tissue samples, even if there is no immediate plan

to use them, with the aim of tissue sharing with collaborators or use in future experiments within our own research if required. A few limited studies in the literature describe differences in phosphate homeostasis in male's vs female's, our studies will therefore where appropriate use both sexes to further investigate this possibility.

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 protocols described have been designed to be the most refined possible, using the minimum number of animals, to provide statistically satisfactory results. These protocols have been planned to cause the least pain, suffering or distress whilst adequately addressing the scientific question they have been designed to answer. One example of this is the use of streptozotocin for the induction of type 1 diabetes. The original method used fasted animals and was associated with early hypoglycaemia-related mortality. Using fed animals massively reduces this risk and in 25 years of using STZ in fed animals I have not had any mortality associated with administration of the drug.

In most instances, the rat is the animal of choice as surgical or chemical induction of chronic renal failure or diabetes is generally easier and more reproducible in rats. Rats are also recognised to be less susceptible to stress and weight loss when placed in metabolic cages. However, genetically modified mice, such as those lacking the gene for a specific transporter or regulatory protein may also be used under this licence.

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

Non-mammalian animals are limited in their use because they do not have the same complex interactions involving different hormones and organs that are used to maintain ion homeostasis in humans. We can't use embryos or very young animals as the control of phosphate homeostasis is different at different stages of development. Importantly, we are most interested in what happens in adults as this is when kidney disease and the associated cardiovascular disease, or elevations in blood pressure most commonly develop.

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 harm to the animals. All animals are routinely monitored for adverse health issues. To minimise the harm 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 body condition score sheets for animals undergoing long-term procedures and have clear criteria set out in the experimental plan regarding humane endpoints. This allows the researchers and animal staff to identify potential issues with individual animals, enabling any animal that approaches the humane endpoint to be humanely culled as early as possible.

Animals requiring repeated handling, such as those administered a substance by oral gavage, will the handled regularly before the procedure starts in order to reduce stress.

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

We will follow 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

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 are regularly informed of regional 3Rs symposia and receive information from NC3Rs regarding advances in the 3Rs. We will regularly check information on NC3Rs website and have signed up for the NC3Rs newsletter. 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, and our collaborators do the same.

38. The study of rejuvenation through modulation of senescence and reprogramming

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, Reprogramming, Fibrosis, Cancer, Aging

Animal types	Life stages
Mice	adult, juvenile, pregnant, 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 delve into the reasons and mechanisms behind aging, and to see if we can counter this process. This could be achieved either by eliminating cells that have aged beyond a point where they may now be harmful to our body, known as senescent cells, or by inducing old cells and tissues to behave like young ones again, a process referred to as cellular rejuvenation or cellular reprogramming. These processes of senescence and rejuvenation are also observed in various disease conditions, such as during tissue damage and aging. We plan to examine the impact of these states and processes at three different levels: the individual cell, the surrounding tissue, and the entire organism. Our goal is to gain a deeper understanding of these processes, both individually and in their interactions, and to use this knowledge to rejuvenate cells, tissues, and whole organisms, thereby reversing 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?

Aging and rejuvenation research has become a critical area of scientific inquiry with significant implications for society, particularly as the global population continues to age. Aging represents a major risk factor for the development of a number of diverse diseases such as tissue fibrosis, cancer, cardiovascular disease, and neurodegenerative disorders. These conditions are not only devastating for individual sufferers, but also impose a significant and rising cost on society. Our research looks to understand the underlying mechanisms of aging and age-related diseases, the associated changes in cellular states, and to identify interventions that slow, prevent, or reverse these disease-related states, ultimately supporting healthy aging.

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

The aim of this project is to enhance our understanding of various disease conditions linked to senescent cells and aging. Importantly, this will allow us to embark on innovative studies that aim to alter the progression of disease or reverse these conditions. In the short term, our outputs will involve the creation of new knowledge that will be shared through publications and conferences. For applications that have commercial sensitivity, we will also work on developing patents for the treatment or prevention of chronic diseases, with the ultimate goal of extending the health-span. Health-span refers to the period of one's life that is spent in good health, free from serious diseases or disabilities.

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

Both short- and long-term benefits are predicted.

In the short-term scientists will benefit from an increased knowledge of basic science in the fields of senescence, aging and cellular reprogramming. The majority of academic knowledge in the fields of senescence and cellular reprogramming has been produced in a tissue culture plate in a small number of cell lines. However, we also know that there are cell-type specific responses to these cellular processes (i.e., heart cells may react differently than liver cells) and that the interaction between different cell-types (e.g., immune cells and liver cells) can alter the outcome. As such it is imperative that we try to model these. As such we intend to publish as much as possible for the wider community once enough data is gathered. Additionally, a large portion of our research is conceptually novel, and will open up new lines of research in the senescence, aging, and reprogramming fields.

In the longer-term we hope that society at large should benefit from our discoveries as our conceptual academic research is validated in further animal models, in human clinical studies, and where possible provide a broad impact on human health-care.

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

Our lab is very well established the fields of senescence, cancer, reprogramming and aging. We collaborate extensively within and outside of our geographical location. We will continue to collaborate extensively, sharing expertise when requested, as well as primary data, protocols, and knowledge through publications. Additionally, we always strive to include negative data in publications as we believe this information is as informative as positive data.

Large datasets will be disseminated through open access repositories, online sites that any researchers will be able to access to download and use the raw data for their own purposes.

Communication of significant findings will be enhanced with support from dedicated public relation teams, in addition to social media platforms.

Species and numbers of animals expected to be used

- Mice: 18080

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 models play an essential role in preclinical and translational research. In this research we will employ both wild-type mice and genetically engineered mouse models. The use of genetically engineered models here will be dependent upon the specific scientific question and aim, e.g. to enable the removal of certain immune populations, to enable the expression of genetic factors, or to block or to turn on a cellular pathway in a cell-type or organismal-wide manner.

From an experimental perspective, our studies are focused predominantly on ageing, chronic disease, and senescence, as such we prerequisite adult mammalian organism with fully developed organs, as well as require the use of aged mice, to allow us to characterise how different tissues and organs change and deteriorate with the passage of time. However, at a conceptual level ageing is a continuation of the developmental process and we also look to take samples from young mice to understand this process in its entirety.

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

Broadly our experiments fall into one main thrust; to induce a dysfunctional tissue state and to characterise the response of the tissue and how this changes with age. Mice will then have particular genetic or pharmacological interventions to promote efficient repair or rejuvenation. This will often include a tissue damage induced by a chemical, transgene induction (viral or genetically encoded), blood sampling to determine tissue functionality based on serum markers, and pharmacological interventions to promote rejuvenation prior to, during, or after tissue damage.

Typically tissue dysfunction will be achieved through natural means (i.e., aging), metabolic interventions (e.g. high fat diet), or pharmacological intervention. For the latter this will be achieved through intratracheal delivery of damaging agents for lung damage, IP injections for liver and pancreatic damage, oral administration for intestinal or esophageal damage.

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

Many of our tissue damage models will induce alterations in weight and in some situations transient pain. Some of our experiments are long term, attempting to understand how

cellular populations change after our interventions however, the tissue damage and associated pain will generally be short- term in nature.

Colon damage: Mice will be expected to transiently lose weight and undergo some transient discomfort

Lung damage: Mice will be expected to transiently lose weight and have alterations to breathing rates. Breathing and weight loss should stabilise over the course of 14 days, representing damage induction and recovery period.

Pancreatic damage: Damage to the pancreas will result in weight loss and transient discomfort for the mice. During the process they may be given pain-relief to minimise the effects.

Chronic Liver damage: Mice will either have drug damage to the liver, or metabolic damage (e.g., a high fat diet). The establishment of liver disease should not result in any obvious impacts on the animal. In a small number of long-term mice these may also result in tumour development.

Acute Liver Damage: Mice will undergo an acute liver stress which will result in liver damage and fatigue in mice during the first 24 hours.

Rejuvenation (genetic): Most mice will show no effects but some mice will lose weight during this process as the colon and pancreas become less functional whilst they rejuvenate. This process usually lasts for a week and mice regain weight once completed

Rejuvenation (drug): Our intention is to mimic aspects of genetic rejuvenation with well known and published with drugs, as such we expect minimal side-effects

Kidney damage: damage to the kidney will result in an acute damage that is not expected to produce more than transient discomfort. A low percentage (<10%) of mice may display signs such as peritoneal guarding.

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: 53%
Mild: 20%
Moderate: 27%

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?

We perform many experiments in non-animal models however the interactions of different cellular populations within a tissue are currently too complex to recapitulate in a culture dish; for this reason it is essential to carry out some experiments on animals. This is particularly true in the context of ageing and senescence models where our ability to replicate these in cultured conditions is limited.

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

We actively use and/or intend to use non-animal alternatives. We attempt to use all the methods synergistically, gaining the maximum benefit from each and reinforcing the strength of our findings. These alternatives include:

(1) cells grown in petri dishes

(2) Organoid cell models; where we try and force cells to grow in 3-dimensions like mini tissues, as opposed to a flat sheet of cells in a petri dish

(3) Tissue cultures; where we take organs and slice them into thin sections to keep in incubators. One mouse organ can be sliced many times to reduce the numbers of mice we need to answer a scientific question

(4) Modelling via computational approaches

Why were they not suitable?

Each of the models described above has advantages over animal models including a reduced financial and time cost, as well as an ethical benefit. However, these models are limited in their ability to fully recapitulate tissue states and the interactions between tissues. Additionally, models that require proliferation (cell culture or organoid models) are not well suited to aging models which usually have defects in the capacity to proliferate.

Where possible we will use these systems as they can help inform the correct types of animal models and experiments. We will also do the reverse and take our research findings from animal models and try to build better non-animal models that recapitulate these settings. We do not like to call these unsuitable models, but instead believe that these models can act synergistically with 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 lab has been maintaining mouse colonies and performing mouse experiments for the past 20 years. The estimated number of animals we will use is partly based on our usage over the previous 5 years and our plans moving forward. The numbers of mice that form our experimental cohorts are carefully considered to make sure we can answer our

scientific questions which have specific end- points. These may be histological in nature, or -omic based.

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

We used a combination of our own historical data and experimentally obtained to help predict sample and effect sizes where possible. Addition we have previously used the NC3Rs experimental design tool to assist in study designs. Our current protocols are designed to enable us to obtain as much information (tissues, blood sampling, sequencing) from each experimental cohort as possible, reducing the need to re-run studies to acquire additional samples.

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 research, we often use organisms that have been genetically altered to have multiple versions of a certain gene. Because of the way genes are passed down (a principle called Mendelian inheritance), we sometimes end up with extra animals that don't have the gene mix we need. We are always trying to improve our breeding methods to get the exact types of animals we need for our experiments. We are also exploring other ways to make genetic changes, like using viral delivery mechanisms (AAV, lentivirus) or techniques (like LNP-mRNA delivery and CRISPR). These methods let us add or change genes without having to breed animals with those changes.

We will also run smaller "pilot" studies to help us understand the real distribution of results of what we are studying, and we will consider factors like the sex of the animals which may enable us to reduce variance. This is true particularly for tissues such as liver which is known to display sexual dimorphism for some phenotypes.

Lastly, we will collect and save as many samples as we can from each experiment. This will help us build a library of tissues we can use in future research. This not only reduces the number of new experiments we need to run, but also allows us to share samples 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.

Lineage Tracing: These mouse models use special markers to 'tag' cells of interest so we can then watch how they change and grow. This helps us understand things like how the cells look, how many there are, and what they do. To label the cells we must use particular substances to help with this process. These can be given in food, water, or by injection. These have a minor impact on the mice and can be done before, during, or after we damage or modify tissues.

Yamanaka Factor Inducible Models: These drug inducible models provide tissue specific (e.g., just the liver) or whole body activation of 4 genes known as the Yamanaka factors. These models also enable us to titrate the dose. The higher the dose the more effects seen on the mouse, leading to phenotypes such as loss of weight. However, with these models, the higher dose is also associated with more rejuvenation phenotypes. For the majority of our studies we will be using low, medium and high doses. The higher doses leading to greater effects, such as weight loss, and the low to medium doses leading to little to or no change in weight. In addition to these models we will also be using viruses and Lipid Nanoparticle delivery of these factors to minimise the potential side-effects (e.g., weight loss).

Progeria Model: This is a model we use to study a disease called progeria, which makes people age faster. Progeria mice have symptoms similar to this disease and our aim is to use these models to understand progeria and aging and trial therapies we believe will alleviate this condition. Unfortunately there is no alternative that is associated with less pain/distress, however with good husbandry practice we will be monitoring these mice closely to ensure they are experiencing the least pain/distress possible for the shortest period. This includes extra checks and monitoring.

Tissue Damage Models: Here we will use well validated and widely used models to induce tissue damage transiently in the liver, lung, colon and pancreas. In our pancreatic tissue damage model we will aim to perform a low level pancreatitis induction which leads to a long term change to the tissue, before inducing pancreatitis again. This re-challenge model is required to see if we can reset the tissue response in the intervening period.

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

Using animals at an immature life stage is not appropriate to our studies due to the fact that we are looking to study aging and chronic disease development. Additionally, terminally anaesthetised animals cannot be used as our experiments are conducted over days to years.

For the study of tissue damage, aging, and cell fate we require the use of mammalian organisms with fully developed organs.

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

Increased monitoring will occur during periods where there is an increase in the welfare cost to the animal e.g., during tissue damage, and also as an animal ages. Aged mouse colonies can be difficult to manage as individuals can succumb quickly, as such we will implement a cage monitoring system based on a combination of clinical signs and mice will have enhanced monitoring and checks with increasing age. In addition to this, husbandry based refinements will be employed where possible e.g., food on the floor of cages for mice with limited movement.

For drug studies we will always look to use the least invasive route for drug administration, and where testing drugs or combinations that we have no previous personal experience with we will always perform initial pilot studies before embarking on a larger studies.

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

When planning experiments, we will refer to the PREPARE guidelines and for general guidance in our experiments we will adhere to the LASA guidelines.

For aging studies there are a number of studies we will consider as best practice guidelines such as: "A toolbox for the longitudinal assessment of healthspan in aging mice" (Bellantuono I. et al. Nat

Protoc. 2020), "Aging Research Using Mouse Models" (Ackert-Bicknell CL et al. Curr. Protoc. Mouse Biol. 2015), and "A clinical frailty index in aging mice: comparisons with frailty index data in humans" (Whitehead et al. J. Gerontol. A Biol. Sci. Med. Sci. 2014).

Additionally, we will use NC3Rs guidelines on breeding to minimise wastage, as well as conforming to the home office “Efficient breeding of genetically altered animals assessment framework” to ensure best practice when maintaining colonies.

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

We currently receive regular updates from the NC3Rs newsletter and will strive to have regular discussions with the Named Persons at our facility to ensure we are kept abreast of any major updates that may impact our work. Where possible if new protocols or refinements are reported to offer superior advancements in the field of the 3Rs we will look to implement them within our own studies, unless doing so would invalidate the results of an already established project. In this scenario, once the existing project has completed, any new projects that require the same technique would implement the new protocol.

Importantly, we will also attend conferences and strongly believe in proactive discussion with groups performing experiments in the same or related fields both locally and at geographically distant locations. Here sharing experiences and protocols is essential for ensuring we can achieve better scientific data with fewer mice using more refined techniques, and enables us to be kept abreast of any new techniques and technologies.

39. Neurodevelopmental and Neurodegenerative Disease Mechanisms 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
- 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

Autism, Schizophrenia, Tourette's syndrome, Chemo-brain, Drug development

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant
Rats	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 aims of this project are to understand how genetic and environmental risk factors for neurodevelopmental and neurodegenerative disease impact on brain functioning and behaviour, and to utilise preclinical rodent models to develop and validate improved treatments for these 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?

Neurodevelopmental and neurodegenerative diseases place a huge burden on the NHS and collectively feature top of the World Health Agenda in terms of Disability Affected Life Years (DALYs). The causes of these diseases remain poorly understood and current

treatments are only partially effective. We urgently need to develop new drugs to help people with these disorders. Gaining a better understanding of how genetic and environmental factors contribute to causing these diseases is crucial to helping patients lead a more normal life. Ultimately drugs need to be validated in relevant animal models before their efficacy can be tested clinically in patients with these diseases.

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

This work will provide new insight into how genetic and environmental risk factors increase the risk of developing neurodevelopmental and neurodegenerative disorders. The information generated will also give insight into drugs that may be useful in the treatment of these disorders. A major benefit of this work will involve the dissemination of these novel insights to the wider scientific community, including academics and colleagues in the pharmaceutical industry, through the publication of scientific papers and by presentation at relevant scientific meetings. As a primary aim of this work is the validation of new rodent models for these disorders, in addition to our own work, in the future these models may be used in the pharmaceutical industry for drug validation studies. This work also contributes to the increasing scientific knowledge of these disorders, and towards the more rapid development of effective drugs for their treatment. In addition, these new insights will be disseminated publicly, to improve societal knowledge of these disorders.

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

The academic research community, interested in understanding the mechanistic basis of neurodevelopmental and neurodegenerative disorders, will be primary beneficiaries of this work in the short-term. Researchers within the pharmaceutic industry may also benefit in the short-term, as this project validates relevant animal models and experimental approaches for the drug development process for these disorders. In the longer term the ultimate beneficaries of this work may be patients themselves, as we aim to validate drugs that can in future studies be tested clinically for the treatment of neurodegenerative and neurodevelopmental disorders. In the shorter term these patients and the general public may also benefit from the information this project reveals regarding the mechanistic basis of these disorders.

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

We will actively collaborate with other researchers using complementary approaches to maximise the outputs generated in this project, including through the sharing of available experimental data and animal tissue. We will disseminate the new information produced to academic and industry colleagues through the publication of scientific papers and by presentation at relevant scientific meetings. We will also engage with the public through press releases and public engagement events. We will also share our developing knowledge with other in vivo researchers through presentations, training sessions and research collaboration.

Species and numbers of animals expected to be used

- Mice: 4000
- Rats: 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.

We are using animals that have genetic changes, or have experienced environmental influences, that increase the risk of developing psychiatric disorders (such as schizophrenia), neurodevelopmental disorders (such as autism) or neurodegenerative diseases (such as Alzheimer's). We are using genetic rodent models based on risk factors for these disorders as they provide models with high construct validity. We are using rodents in particular as they demonstrate cognitive processes that are relevant to those that are disrupted in these disorders, unlike simpler non-vertebrate models. As we are interested in mapping the developmental trajectories of these disorders across the lifecourse, from early development and in aged animals, we characterise animals at a number of different life stages.

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

We will utilise animals with genetic alterations that are associated with the development of psychiatric, neurodevelopmental and neurodegenerative diseases in humans. These genetic changes may be present throughout life, or may be induced at a specific age by drug treatment. We will also expose animals to environmental manipulations that are associated with these disease, for example chemotherapeutic agents that induce neurodegeneration and cognitive problems in cancer patients. The chemotherapeutic treatment regimes will be sub-chronic, typically lasting no more than two weeks.

We will observe the behaviour of animals, to determine if they show disease-relevant changes in behaviour that relate to the key symptoms seen in neurodevelopmental and neurodegenerative disorders. For example, we will look at their social behaviour and their ability to complete tasks that look at learning and memory. We will also image the animal's brains to see if they show functional changes similar to those seen in patients. Once these alterations have been identified we will test the ability drugs to reverse these alterations, to determine whether the drugs may be useful for the treatment of these diseases. The brain imaging study that we utilise does not require surgery, but does require a single tracer injection into the intraperitoneal cavity. Drug treatment regimens may be acute, sub-chronic, or chronic, depending on the drug of interest and outcomes being assessed. The shortest drug treatment regimen possible that will allow the detection of positive outcomes will be utilised, informed by existing literature and pharmacological data, where available.

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

The genetic alterations associated with these disease can have negative consequences for the animals, but these typically have very little impact on general animal health. The genetic and environmental manipulations we employ generally have subtle effects on behaviour that must be revealed through the application of specific behavioural tests, to reveal abnormal behaviour in the animals. In some behavioural tests we have to restrict the animals access to food, so that they are motivated to work for a reward. This means that animal may lose weight relative to their weight when they have unlimited access to food, but we ensure that this is limited in magnitude and duration. We closely monitor animals to make sure they don't lose too much weight and remain healthy. Many of the behavioural tests we employ characterise behaviours that are naturally expressed by animals, and these involve subthreshold levels of suffering.

The administration of substances by subcutaneous injection can result in transient mild pain and discomfort, while dosing intraperitoneally carries the risk of organ puncture and peritonitis.

Administration of substances by oral gavage carries the risk of installation to the lung and irritation of the throat. Administration of substances by highly trained and competent individuals substantially reduces the risk of these adverse events occurring, but if they do arise we will ensure that they are limited in their duration. The substances we administer to animals can also have negative consequences. However the substances we will use are generally well characterised and we will employ the lowest dose and duration of administration to limit the impact on animal welfare and to avoid any potential toxic effects.

Substances we will administer include immunogenic compounds (including PolyIC) and chemotherapeutic agents that can induce transient (less that 24 hours) symptoms of illness. Chemotherapeutic agents can also induce weight loss, so we employ a dosing regimen to ensure that this is limited in the animals. The acute administration of substances that modify the function of the brains neurotransmitter systems can induce short-term changes in behaviour and body temperature (normally less than 6 hours in duration). While chronic administration of substances, such as antipsychotics, can induce changes in body weight (both increases and decreases), we set strict limits in terms of the decrease in body weight and the impact on general animal health that is allowed.

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 animals will experience severe side effects.

5-10% of animals may experience a moderate severity. 60-70% may experience a mild severity.

20-35% of animals will experience sub-threshold 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?

Neurodevelopmental and Neurodegenerative disorders are complex diseases, often characterised by very subtle dysfunction in the interconnections that exist between different brain regions. Rats and mice are the lowest vertebrate groups that can be used to reproduce the complex neurobiological and behavioural deficits, particularly the high-level cognitive deficits, of the neurodevelopmental and neurodegenerative disorders. Assessment of the ability of drugs to successfully restore the abnormal patterns of neurochemistry, brain functioning and behaviour also necessitates the use of rodents, as the well-defined functional organisation of the brain and neurochemistry in rodents is closely aligned with that in higher order vertebrates and humans. Work validating novel compounds for the treatment of neurodevelopmental and neurodegenerative diseases ultimately need to be tested in rodent models prior to their assessment in clinical trials.

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

In vitro model systems (e.g. cell culture assays) and non-regulated experimental animal studies (e.g. fruit flies and worms) can be informative in the context of this project, and we regularly utilise these model systems in our studies to gain valuable information that informs our rodent studies. However,ultimately the aims of this project require the use of rodent models as these translationally-relevant outcomes can not be adequately assessed in these other model systems.

Why were they not suitable?

The subtle and complex alterations in brain connectivity and functioning seen in neurodevelopmental and neurodegenerative disorders can't be adequately replicated in cellular models or in nonmammalian experimental animals (e.g. Drosophila melanogaster). Non-mammalian experimental animals often lack the genes/genetic fragments of interest in our studies and lack the complex cognitive abilities that are of primary interest in our 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?

We have used published, in-house and pilot data to design our experiments. Experimental design is reviewed regularly when new data becomes available. Animal numbers are based on our previous experience of undertaking projects that are similar in scope and duration to this one. As we typically breed our genetic experimental animals in-house a number of animals have been allocated for this purpose.

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

We have used our experience from previous studies and the NC3Rs experimental design assistant (EDA) to determine the number of experimental animals required. The proposed experiments and methods of analysis have been considered and have been discussed with an independent local statistician.

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 will be conducted, recorded and published according to the ARRIVE guidelines. Our breeding strategies will be designed to generate the minimum possible number of non-useful animals in accordance with LASA guidelines to minimise the production of surplus animals and following the ASRU code of practice. All the mouse lines in this study will be imported from other establishments.

We will measure production and breeding performance and ensure the minimum numbers of animals are used for breeding and maintenance. We will use inbred mice (inbred mice

are as closely related to one another as is possible) which reduces inter-animal variability and thus overall numbers required.

We have carefully calculated the minimum number of mice required for the experiments described in this project.

If relevant published data are not available, preliminary drug evaluation tests are conducted using in vitro assays, where possible. Only compounds that show significant activity in vitro, or that have proven efficacy in the published literature, will be investigated in vivo.

Wherever possible, behavioural, brain imaging and neurochemical studies will be carried out using the same animals. This will both increase the value of the information gained and reduce the total number of animals required for these studies overall.

Experimental variability is minimised through the implementation of standard housing conditions, standardized breeding and animal handling procedures and through the use of experimental standard operating procedures.

Breeding of genetically modified mice can involve relatively large numbers of animals. However, in our studies we generally employ heterozygous mice, particularly where these are of greatest translational relevance, which reduces the number of animals used. For homozygous mutants, the numbers used will be minimised by breeding separate transgenic/knockout and wild-type lines, wherever this is possible and where it doesn't compromise the experimental outcome. New lines will only be initiated where there is strong evidence that the gene concerned is linked to neurodevelopmental or neurodegenerative disease.

My lab has an ongoing commitment to the reduction of animal use in the context of behavioural longitudinal experiments, with previous work being undertaken by a PhD student funded by the NC3Rs aimed at developing novel statistical approaches to reduce animal usage in these types of studies (https://nc3rs.org.uk/developing-novel-experimental-design-and-analysis-model-longitudinal- animalstudies-high-dimensional). My lab also has an ongoing commitment to the refinement of animal use, explified by the development of a less invasive functional brain imaging protocol that reduces animal suffering, and our ongoing MRC National Mouse Genetics Network (NMGN) project (MURIDAE) that aims to characterise behavioural deficits in transgenic mice by continuous behavioural monitoring in the homecage environment.

We regularly share tissues that we can not use in our studies with other researcher groups to minimise animal use and maximise the experimental insight 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.

The genetically modified strains we use do not generally have overt behavioural phenotypes and exhibit only subtle behavioural deficits that are evident only under specific testing conditions. The precise adverse effects of novel genetic alterations that may be investigated in the future are not known at present. Some knockout lines may be embryonic lethal or lethal before adulthood, and such lines will be utilised as conditional knockouts if available, or maintained as heterozygotes. As a general rule we employ heterozygous mouse models in our experiments as the majority of genetic alterations associated with neurodevelopmental and neurodegenerative disorders tend to be heterozygous in nature. Thus we employ heterozygous models, when most appropriate, to maximise translational relevance and these tend to display phenotypes only under specific testing conditions. We will also employ conditional knock-down models to mitigate unwanted neurodevelopment effects, when relevant.

We have found that repeated phencyliding (PCP) treatment in rodents produces a pattern of metabolic imaging, biochemical and behavioural changes that mirror those observed in the brains of schizophrenic patients. We have recently observed similar changes in the brains of mice with a targeted mutation in genes strongly associated with neurodevelopmental and psychiatric disorders.

Hence the use of rats and mice can produce phenotypes with a high degree of translational value. To ensure translational validity we will employ models with a strong rationale in relation to disease aetiology, using models based on established genetic and environmental risk, or established neurochemical dysfunction. The models of neurodevelopmental and neurodegenerative disease employed in these studies are such that only relatively subtle behavioural phenotypes are anticipated. Thus the models themselves are unlikely to display overt signs of discomfort.

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

As we are assessing animal behavioural performance in complex cognitive tasks , that have translational relevance to the cognitive problems experienced by patients, we can not use species that are less sentient. Due to the developmental onset of neurodegenerative diseases and the importance of ageing in the manifestation of neurodegenerative diseases we can not use animals at more immature life stages for these studies. Terminal anaesthesia would impair brain function in the animals, and would not allow for behavioural testing.

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

We will ensure that the least invasive methods of dosing are applied, including the use of anaesthesia for humane restraint when appropriate.The doses of drugs employed will be selected to be at the lowest dose possible to cause measurable effects without causing toxicity and will be based on published data, where available. This will minimise any potential discomfort. In any case where there is little available information on the in vivo effects of a compound pilot studies will be employed using incremental doses and very small group sizes prior to the initiation of a full experiment.

Where previous experimental work can be used to inform the presence of translationally-relevant phenotypic and endophenotypic changes in experimental models, we will utilise the earliest translationally-relevant testing point in the lifespan of the animal and employ the minimum length and frequency of behavioural testing to obtain meaningful results.

In our studies we employ a refined, updated method of functional brain imaging that reduces the suffering of animals, as the protocol used does not require the surgery (intravenous cannulation) or prolonged restraint necessary in the original protocol. When food restriction and pro-inflammatory protocols are employed we employ enhanced welfare monitoring approaches to ensure that suffering is minimized.

Animals under procedure will be closely observed, clinical signs (including hunching, grimace, normal movement, body temperature) will be monitored to determine when the humane endpoint has been reached and will be adhered to at all times to minimise harm.

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

We will follow best practice guidance as provided by the nC3Rs to ensure that experiments are conducted in the most refined way, including guidance provided as part of the Experimental Design resources (https://www.nc3rs.org.uk/experimental-design), the PREPARE guidelines (https://norecopa.no/prepare) and by reporting our experimental data in accordance with the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines). During food restriction and pro-inflammation protocols we employ both weight monitoring and body score condition monitoring based on published protocols. For pain assessment we refer to the nC3Rs mouse and rat grimmace scales.

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

We regularly monitor the NC3Rs website (https://www.nc3rs.org.uk/) for any news regarding advances in animal welfare and for any relevant events being held. We will also regularly attend scientific meetings with a strong 3Rs focus, including meetings held by the British Association of Psychopharmacology (BAP) and British Neuroscience Association (BNA). We also regularly share best 3Rs practice with University colleagues through participation in the Universities Animal Welfare and Ethics Review Board (AWERB). We regularly implement new 3Rs advances into our experimental studies, wherever possible. Through our involvement in the MRC National Mouse Genetics Network (NMGN) we will share best practice and advancements on 3R principles with colleagues from across the UK.

40. Modelling cardiovascular disease, tissue repair and heart regeneration in zebrafish

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cardiovascular disease, Regeneration, Zebrafish, Inflammation, Tissue repair

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 purpose of the outlined work is to advance understanding of the cellular and genetic processes involved in tissue healing with the ultimate aim of facilitating the development of interventions that could be used clinically on humans to restore the normal appearance and function of tissues, including those of the skin and heart, following 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?

For humans, the loss of function and often disfiguring scar tissue that forms following injury or disease is a major cause of suffering and disability. Burns, both heat and chemical, are particularly problematic, causing serious disfigurement and loss of function whilst the scar tissue that forms following a heart attack results in permanent loss of function, often leaving the patient with disabling cardiac insufficiency. Unlike mammals, many fish, including Zebrafish, are able to restore full function to tissues following injury. They achieve this by resorbing the scar tissue that initially forms following injury and replacing it will fully functional tissue. The purpose of the outlined work is to advance understanding of the cellular and genetic processes involved in the healing of skin and cardiac tissues in Zebrafish. In so doing, the work will advance understanding of how the processes involved

in healing and tissue repair differ between fish and mammals with the aim of identifying interventions that could be applied to improve healing outcomes in humans.

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

The primary output of this project will be new data that advances understanding of the cellular and genetic processes involved in tissue repair and healing of skin and cardiac muscle in Zebrafish. These findings will be published in peer-reviewed scientific journals. All papers will be published under an open-access policy meaning they can be read by everyone. We will also attend scientific conferences to report our findings and conclusions, and relay these to the general public whenever possible.

Examples of public events where we have already discussed our work: 1 - presentations at the annual nationwide “Pint of Science” festival, 2 - giving a departmental public lecture, 3 - speaking at multiple charity supporter events for the BHF and Scar Free Foundation, 4 – media coverage of our Scar Free Foundation grant award.

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

In the short term, the beneficiaries of the work will be other scientists working to advance understanding of the cellular and genetic processes involved in wound healing. In the medium term, the work is expected to benefit scientists working to develop ways of modulating the healing process in humans to improve the appearance and function of tissues damaged either by trauma or disease. In the longer term (approx. 10 years e.g beyond the time scale of this project), the contribution of the work to the development of interventions that enhance tissue repair, is expected to benefit patients,clinicians and health care providers (such as the NHS). This is likely to take the form of using our zebrafish models to test the feasibility and functionality of new therapeutic strategies (as we have already published with collaborators in synthetic biology), our findings identifying new inflammatory therapeutic targets to aid healing and our new disease models allowing insights into disease mechanisms and providing new platforms for therapeutic screening.

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

The work will be conducted in collaboration with an established network of scientists working in related fields of research. All findings (positive and negative) will be published in peer reviewed open access scientific journals and presented at scientific conferences and at talks given to the general public.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 14,900

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.

The purpose of the outlined work is to advance understanding of the processes involved in tissue repair and healing in fish with the aim of facilitating the identification of interventions that could be used to improve the outcome of the healing process in humans. Zebrafish provide the model system of choice for these studies because, 1) unlike humans, they can

fully regenerate the cells of their skin and heart following injury, thereby restoring both normal function and appearance, 2) they are easily maintained under laboratory conditions, 3) methods to manipulate their genome are well established, enabling subsets of cells to be marked or modulated to enable their role in tissue repair to be determined, 4) the transparent nature of the skin of zebrafish enables the healing process to be visualised in a manner that is not possible in most other species, and 5) the expertise to undertake the work are already well established within my research group. Adult zebrafish, and sometimes aged fish, will be used for most studies because the immune system of immature fish is not fully developed and is therefore less relevant to the healing process in humans.

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

The majority of fish (95%) used in the outlined studies will be used for breeding and will carry genetic modifications that either modulate the function of specific cell subsets and/or enable them to be identified e.g. through the use of fluorescent markers. These mutations are not expected have any adverse consequences for the wellbeing of the fish. Some of these fish (5%) will be allowed to grow to old age before being used. The majority of the fish (75%) will not undergo any procedure and be killed using a humane method to enable tissues to be harvested for assessment using non-animal laboratory based assays. A small proportion of the fish will carry harmful mutations that predispose to adverse effects in later life, but these fish will be used at an age before adverse effects are expected to occur.

Approximately 20% of the fish will be used in tissue injury experiments in order to study the role of specific subsets of cells in the healing process. Most of these fish, including some that have been naturally aged, will carry genetic mutations that do not impact upon their wellbeing. All fish will receive a small tissue injury following the induction and maintenance of general anaesthesia (typically, a ~2mm diameter injury to the skin of the fin or flank made using a dermatological laser or a small cryogenic injury to the muscle of the heart). The fish are expected to make an uneventful recovery following injury and are not expected to show any overt signs of suffering. Some of these fish will receive treatments that modulate the healing process via their tank water, but these treatments are not expected to impact negatively upon their wellbeing. Some fish will undergo imaging under terminal anaesthesia. At the end of the study period, all fish will be killed using a humane method so that their tissues can be harvested for analysis.

A small proportion of fish used (5%) will be generated with genetic mutations associated with cardiovascular disease in humans. These mutations are not expected to have any adverse effect until the fish are old and the fish will be carefully monitored and killed before they reach an age at which harmful effects are expected to occur. Following killing, tissues from these fish will be harvested for assessment using tissue-based ex-vivo laboratory assays.

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

No adverse effects are expected for the vast majority of fish that carry genetic mutations that are used for breeding. Some of these fish will be maintained until they are beyond their normal reproductive age, however, they will be killed before they reach an age when overt signs of senescence are expected to occur. Approximately 20% of the fish, including aged fish, will be used in tissue injury experiments. In all cases, tissue injury will be performed under general anaesthesia and the injury induced is very small. Whilst it is perceivable that fish undergoing injury will experience some pain upon recovery, the vast

majority are expected to recover uneventfully and to show no detectable signs of suffering. Any fish showing overt signs of suffering, such as abnormal or impaired swimming, will be immediately killed. At predetermined timepoints following injury, some fish will be anaesthetised so that imaging can be undertaken, no adverse effects are expected as a result of imaging and the fish are expected to recover uneventfully from the anaesthetic and to continue to swim, feed and behave normally.

A small proportion of the fish (<5%) will be bred with genetic mutations associated with cardiovascular disease in humans, however, these mutations are not expected to have any adverse effect until the fish are old. These fish will be carefully monitored and killed before they reach an age at which harmful effects are expected to occur.

In all case, the fish will be killed at the end of the study using a humane 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)?

Mild 75%
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?

The healing process of living tissue involves many complex interactions between numerous cell types and a plethora of molecular processes, many of which are at only partially understood or have yet to be discovered. Consequently, due to the porosity of current understanding, it is not as yet possible to truly replicate all of the processes involved in wound healing using either cell culture or computer simulation. Nevertheless, if possible, cell culture systems will be used to study aspects of the healing process. Cell culture can be a useful “reductionist” system where we can investigate molecular interactions between specific cell types identified in whole animal studies. For example, we are interested in how two cell types can communicate via extracellular vesicles (EVs) and aspects of this communication would be easier to study in vitro.

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

We are always considering alternatives, predominantly how we might use cell culture systems to supplement our work. Unfortunately, not many zebrafish cell lines exist so most in vitro work still relies on using primary cells and so still requires animals to create these cells. However, testing some of our findings (e.g. communication between two cell types via EVs) in mammalian cell culture would be a good first step towards translation to humans. As yet, it is not possible to use computer simulation for our studies owing to the limited level of current understanding of the processes involved, however, we plan to

utilise AI technology to obtain more data from our planned imaging experiments, increasing the amount of data we can get per animal. We already have collaborations in place to establish this work.

Why were they not suitable?

We cannot use cell culture for all of our studies due to the limited cell types we can assess in these kinds of experiments. For example, one thing we study is how extra immune cells are signaled and then recruited into a damaged heart from the blood. Even if we were to culture a whole heart in a dish (which we do for some imaging studies), we cannot study how new cells get brought to the injury in this system. We can only do these kinds of complex studies 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?

We have a lot of experience in designing tissue repair and regeneration studies and so can estimate numbers from this experience. We currently maintain 70 separate zebrafish lines (95% of them GA) in house, which equates to over 3500 fish. This number allows us to keep sufficient breeding age fish of both sexes to maintain each line (e.g. 2 tanks per line of separate generations) and to provide enough fish for our planned experiments. We need to maintain this many lines because we require complex combinations to allow us to perform our planned experiments. Indeed, our most recent publication, addressing part of one objective, contains data on 12 separate lines. As an example, for one mutant line we may wish to study the inflammatory response to an injury (requiring that mutant to be crossed to a double transgenic line labelling two immune cell types) and the movement of the skin to recover the wound (requiring the mutant to be crossed to a separate double transgenic line labelling two skin cell types). We also then require control siblings (wildtype and/or het) to be crossed into the same transgenic backgrounds. During this project we plan to investigate 12 mutant lines that we already have crossed into different backgrounds, and we wish to make more lines for targets that we identify during our project. We also wish to study ageing and regeneration in non-mutant transgenic (GA) lines. The majority of fish will only be used for breeding to maintain these lines (often with tissues harvested when culled), approximately 25% will go into injury or ageing protocols.

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

We routinely run power calculations and statistical analyses on existing data to ensure that we are meeting statistical power but also using the minimum 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?

We routinely take multiple tissues from every animal sacrificed so we can maximise the amount of information from every animal used. This includes animals that have only been

used for breeding that are used for uninjured tissue controls. We often use larval genotyping (pre-5 dpf) methods to reduce the number of protected animals we generate.

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 (75%) of zebrafish required for the outlined studies will only be used for breeding. This number is required to maintain the large number of different lines we require. These fish will only experience some handling, sometimes (but not always) short term anaesthesia and a schedule 1 procedure and will therefore experience minimal suffering or distress.

The fish used for injury procedures are all likely to experience some pain upon recovery from anaesthesia, however, the injury induced is very small and the fish do not show overt signs of pain or suffering and continue to swim, feed and behave normally. The healing process is rapid in Zebrafish

e.g. skin lesions are invariably closed within 12 hours.

Genetic modification techniques will be used to alter the genome of some fish (5%) to model some aspects of human cardiovascular disease. This procedure is performed at the 1-8 cell stage of very early embryogenesis before the nervous system has developed and therefore causes no suffering. Some of the mutations induced have the potential to cause lasting harm however, in all cases, disease is not expected to develop until late adulthood or old age. These fish will be carefully monitored and killed at an age before which overt disease is expected to occur or as soon as signs of disease become apparent, therefore the majority of these fish are not expected to experience suffering and for those that do the severity of suffering is expected to be mild and of short duration.

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

Less sentient, invertebrate species are unsuitable for these studies as their immune system and tissue repair mechanisms differ markedly from vertebrates and are therefore unlikely to provide an insight into interventions translatable to humans.

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

The procedures required to undertake the outlined studies have all been extensively refined during previous projects. The majority of fish (75%) will only be used to create and maintain genetically altered lines. These alterations (e.g. labelling specific subsets of cells with a fluorescent marker) are not expected to have any adverse effects. These fish will be humanely killed so that their tissues can be harvested for analysis and testing.

Approximately 20% of the animals will be used in tissue injury experiments to study the role of specific immune cells in the healing process. Tissue injury will be performed under general anaesthesia and the injuries induced are very small and do not result in any signs of impaired function upon recovery. In the event that a fish does shows signs of impaired function upon recovery (e.g. abnormal swimming motion, isolation from other fish or a lack of interest in food), it will be killed without delay.

A small number of fish will be bred with genetic mutations linked to cardiovascular disease in humans. In all cases, disease is only expected to develop in late adulthood or old age. These fish will be carefully monitored and killed at an age before which overt disease is expected to occur or as soon as signs of disease become apparent, therefore the majority of these fish are not expected to experience suffering and for those that do the severity of suffering is expected to be mild and of short duration.

In all cases, the fish will be monitored daily and more often when undergoing procedures or where there is any concern. Fish will be promptly killed if they exhibit any overt signs of suffering (e.g. abnormal swimming motion, isolation from other fish or a lack of interest in food).

For genotyping of GA fish, we have been trialling the micro-abrasion technique to obtain DNA, rather than using traditonal fin clipping. Initial results have been promising, but we still have some concerns about the reliability of this technique. Once the protocol works robustly in our hands we plan to adopt this method for routine genotyping.

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

The zebrafish research community regularly publishes papers on husbandry and best practice for procedure and these will be followed for the outlined work. Our inhouse Zebrafish group meetings and associated file storage site are excellent ways for us to share new developments and best practice across the whole group, ensuring that we recognise new information in a timely manner. Surgery will be conducted using instruments sterilised in line with LASA guidelines.

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

My institute is committed to promoting the 3Rs and I receive monthly updates on development via our Named Information Officer. I also attend and contribute to 3Rs events organised by my institute and its linked institutes. In addition, my local zebrafish research community (consisting of ~30 people) meet every 2 weeks to discuss research progress and to receive updates from our aquarium facility manager, who regularly attends zebrafish husbandry focussed meetings and reports back on best practice.

41. Exploring The Role of Fibroblast-Macrophage Cross Talk in Arthritis

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

Arthritis, Inflammation, Fibroblasts, Macrophages

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 explore the role of new families of cellular targets expressed on cells that form connective tissue called fibroblasts and a type of white blood cell called macrophages that enhance joint inflammation or promote its resolution during experimentally induced arthritis in mice.

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 now clear that the persistence of inflammation can occur both because of insufficient production of anti-inflammatory mediators as well as the more traditionally established mechanisms of excess inflammatory mediators These observations have important implications for the way in which we treat chronic inflammatory diseases. Much like a diabetic is given insulin to replace the insulin they can no longer make, we could start giving naturally derived anti-inflammatory mediators to individuals with chronic inflammation to restore and replace the ones that they are no longer able to make enough

of. In this way we would be replacing good pathways rather than just blocking bad pathways to restore healthy joints.

Biological therapies (such as anti-TNF) have transformed the lives of patients with Rheumatoid Arthritis (RA). However, their effect is transient, relief is partial and true drug free remission occurs in less than 15% of patients. There remains a pressing need for effective, treatments that really cure as opposed to just treat RA. Our patient partners have explained to us that while current treatments are good at reducing inflammation in their joints, they are not as effective in reducing pain and restoring joint function.

Our preliminary work has identified new molecules on the surface of two major cell types that reside in the joint which regulate joint inflammation and help the resolution of arthritis. They are called macrophages and fibroblasts. However more studies are needed to better understand the cellular and molecular mechanisms involved in how these cell types communicate with each other. In this way we will be able to evaluate the efficacy and safety of reagents that not only block inflammation but also promote the processes that help to resolve it. This will place us in an ideal position to develop new therapeutic strategies that permanently switch off arthritis and ultimately cure inflammatory arthritis such as Rheumatoid Arthritis.

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

The work to be carried under this project will:

- Drive a clearer understanding of how arthritis starts and develops by evaluating the role of a new family of cells and molecules by exploring the cellular mechanisms that trigger inflammation or promote its resolution.

- Reveal new cellular and molecular targets related to fibroblast-macrophage cross talk which will help develop new therapeutic strategies to cure RA.

- Provide new scientific findings on the mechanisms that drive the resolution of inflammation in RA that we will disseminate to the scientific community through oral presentations and posters as well as high impact scientific publications.

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

Short-medium term

The scientific community will benefit from developments and innovations leading to our enhanced understanding of the role of immune effector fibroblasts in inflammatory arthritis described in this licence. Our findings will have an immediate impact on the ongoing projects at the host organisation, and 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 persistence of joint inflammation that we anticipate will underpin the development of the next generation of anti-rheumatic therapies.

Medium-long term

We intend to invite external seminar speakers and scientists who have an interest in the role of fibroblasts and macrophages in chronic inflammatory diseases to our organisation, 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, the scientific community will benefit from developments in 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 and particularly why some individuals with arthritis do not respond to currently available treatments, 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?

Collaborations: This project builds on a strong collaboration between two leading institutions, collaborating with the host institution in the UK. The members of these research teams, with a strong expertise in RA pathophysiology, will provide all the necessary knowledge, skills and abilities to deliver this project and translate the scientific discoveries into patient benefit.

Dissemination of new findings: We and our collaborators are planning to use different approaches to disseminate our research findings and maximise the uptake of our research by the scientific community and clinicians.

- Explanation of project aims and presentation of data at professional meetings and conferences by either oral or poster presentations. This will allow us to raise awareness of our work, exchange knowledge and receive feedback that may be helpful for additional studies.

- Publications of papers in open access peer-reviewed journals to reach the widest possible audience and be available permanently. Negative results will also be published to avoid repeating experiments that are inappropriate for the topic or do not support the initial hypothesis.

Public engagement: Throughout the project, we will engage and involve patients and the public in our research who will participate in the dissemination of our research and provide a powerful voice. Our group has established patient research partners group who arrange regular seminars and events attended by patients with RA to enable the dissemination of our findings into a diverse audience.

Species and numbers of animals expected to be used

- Mice: 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.

Mouse models of arthritis recapitulate some but not all aspects of RA pathogenesis in humans and have contributed directly to the development of new anti-rheumatic therapies. Mice are the lowest mammalian model for human skeletal and immune systems in which we can test the impact of gene manipulation and evaluate the efficacy of therapeutic interventions. We will use adult mice as they have fully developed joints and for fate mapping the origins of fibroblast and macrophage subsets. We will also use conditional gene activation in late embryonic development to establish the developmental origins of the cell types we are manipulating.

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

Genetically altered mice will be bred using conventional methods. Some mutations are not expected to cause any harm until activated by the administration of a substance, typically Tamoxifen. However, one of our genetically altered mouse strains will be susceptible to developing polyarthritis (arthritis where at least 5 joints are affected) and inflammatory bowel disease after the age of 6 weeks. We will keep some males with symptoms to breed and these will receive a therapeutic substance to prevent the onset or progression of the arthritis when they are being used as stud males. Another strain develops arthritis by the age of 3 - 6 weeks. This strain will not go on to be used in experiments but will be humanely killed to allow the harvest and preparation of serum to induce the polyarthritis in other non- symptomatic wild type or genetically altered strains.

Some mice will undergo the induction of Monoarthritis (arthritis with a single joint affected) by up to three injections of an antigen such as Albumin. This will be accompanied by an adjuvant. The first two injections are given via the subcutaneous route with the third one into a knee joint. Some mice will only get a control substance. Typically, this experiment will see a steady increase in the symptoms that peaks at 7-14 days and resolves after 28 days.

Some mice will undergo induction of polyarthritis by two mechanisms. The first includes 2 subcutaneous injections of an antigen into two sites 7 days apart followed by an optional two further injections no later than 21 days after the first via the subcutaneous route. The second mechanism utilises up to 2 injections of serum in the first instance via the 4 intraperitoneal or intravenous route. Arthritis can be maintained for up to 4 weeks with a weekly injection of serum via the intraperitoneal, intravenous or subcutaneous route 2 -5 days post induction. Both methods will be accompanied by an adjuvant. Some mice will not undergo induction as they will come from our breeding protocols and be genetically susceptible as described above. Some mice will only get a control substance. Typically, these experiments last for up to 21 days for the serum induced model and up to 40 days for the collagen induced model. These genetically susceptible mice may develop Irritable Bowel Disease from 6 weeks of age. We will monitor and manage symptoms as they arise.

All animals will have their joints assessed using callipers daily for the first 7 days followed by three times per week to monitor disease progression. This is a non-invasive way to measure the swelling of joints. We will also weigh the mice three times per week and mice will be killed if weight loss of up to 15% is observed. Mice may be given analgesics (e.g. buprenorphine) once mice develop swelling that has increased by 10% during calliper measurements.

Some mice will receive therapeutic substances to see if they improve or slow the progression of the disease. The common routes used for these substances are not expected to cause any adverse effects, however on occasions we may use the intra articular (into a joint) route.

Some mice will undergo a load bearing assessment to see how they use the affected joints. This will be once daily for up to 5 minutes up to a maximum of 4 weeks. This is a non-invasive test which the animal can perform at will.

Some mice will have blood samples taken to enable us to see how the immune system responds to such diseases or treatments.

Some pregnant and adult mice will be given therapeutic agents or have their genetic make-up mapped by being given substances such as tamoxifen. The adult mice that have received the therapies may then go on to have either mono or polyarthritis induced to see how effective the agents are at reducing the adverse effects of arthritis. Pregnant female will not continue in the protocol or undergo this procedure however the offspring from these females may undergo the induction of arthritis.

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

Tamoxifen is expected to cause transient weight loss of up to 15%. This normally is self-resolving once the dosing period has ended.

For monoarthritis disease typically develops within 1-2 weeks. Most mice will experience arthritis for up to 2 weeks. Animals will experience pain and swelling resulting in reduced mobility although they will be able to feed normally and move around. Joint inflammation is defined by redness, pain, heat, swelling, and loss of function. During advanced stages of disease, mice may be given analgesics (e.g. buprenorphine) once mice develop swelling that has increased by 10% during calliper measurements. 10% of mice will be kept alive for up to 12 weeks. If mice are on treatment, there will be very little disease progression. If mice are not on treatment there will be a steady increase in the symptoms listed above that peaks at 7-14 days and resolves after 28 days. Any mice that reach the humane endpoints prior to the 12 weeks will be killed.

When given subcutaneous, some mice may also experience a reaction to the adjuvant displayed as reddening from inflammation at the injection site and in some cases this may become ulcerated.

Ulceration treatment may be initiated by the NACWO/NVS. If the ulcer is not improving within 48 hours (dry and healing) and or >4mm in diameter, the animal will be killed.

For polyarthritis, disease typically develops within 3-4 weeks for the antigen model and within a few days for the serum model. Animals will experience a greater degree of swelling to both the joints and the paws and may experience lameness. Joints and paws will be measured using callipers at least three times per week once the animals show symptoms. As with the monoarthritis there is a risk of a reaction to the adjuvant used with the serum or antigen they are given.

Where we use intra articular injections for our substances, mice may experience lameness. If lameness does not resolve within 24 hours the mice will be killed.

The administration of substances to pregnant mice can damage embryos, cause miscarriage, reabsorption of the embryos or result in obstructed labour. Females will be checked twice a day for 1 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)?

50% Sub-threshold, 12% mild, 38% 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?

To study the inflammatory response, three components need to be examined: time, place, and cell type. While place (organ) and cell type (leucocyte or tissue resident cell) can be examined relatively easily in humans, it is difficult and, in some cases, unethical to perform multiple biopsies, and adoptive transfer experiments in humans with arthritis without underpinning preclinical data that support therapeutic utility. Furthermore, as manipulating fibroblast biology has the potential to affect systemic immune function, we will need to determine whether manipulating fibroblasts as a therapeutic target affects the innate and acquired immune response and this requires testing in different mouse models and those that most closely resemble aspects of human disease. This step is essential to ensure that the translation of our findings to humans passes through the appropriate clinical regulatory stages before use in patients with arthritis. Where possible, human samples (tissues and cells) will be used to test the efficacy of test compounds using in vitro organoid cell culture models.

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

Human primary cells (isolated from human synovium biopsies from the joint) and peripheral blood mononuclear cells (PBMC) isolated from human blood samples will be used for cell isolation and co- culture (organoid) experiments. Organoids are 3D miniature structures cultivated in-vitro with the aim to mimic the structure and function of human organs or tissue. After in-vitro culture, cells and medias will be collected and analysed for genes and mediators known to stimulate or antagonise inflammation and tissue damage.

We are also planning to set up and use multicellular organoids system; 3D spheroids made from synovial cells that are reconstituted with cells derived from synovial tissue for co-culture experiments. Co-culture experiments are an in-vitro model that allows a variety of cell types to be cultured together to study the interactions between these different cell types. This system will provide a powerful tool to study the synovial cellular crosstalk between fibroblasts and macrophages and to test the effect of agents such as antibodies. We anticipate that this parallel approach of in vitro organoid cultures will reduce the use of animal models.

Why were they not suitable?

In addition to in-vitro models, this programme of work requires models that mimic acute and chronic arthritis that recapitulate the temporal aspects of human RA pathogenesis, allowing us to produce biologically meaningful results. This work includes pre-clinical therapeutic (drug and/or cell) efficacy studies required prior to embarking on human clinical trials. There are no other in vitro alternatives to this work.

We will also keep reviewing the scientific literature to identify any new emerging technologies and models that could be potentially adopted instead of in vivo models (such as the 3D organoids mentioned 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?

Animal number calculations are based on data obtained from ours and our collaborators in previous experiments using the same mouse models of arthritis. All experiments are designed to ensure that minimal numbers of mice are used to obtain biologically significant results.

We've used our annual return of procedures data to estimate the number of animals that we will need to use for breeding.

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

Statistical calculations based on previous experiments will be used to ensure that minimal numbers of mice will be used in this project.

When new procedures are planned, we will use the NC3R's experimental design tool for good experimental design. Each animal will be assigned to treatment groups randomly and experiments will be performed blind with respect to the treatment and genotype in to minimise unintentional variability.

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

In vitro methods using human samples will be used initially in all cases to test the efficacy of new compounds and estimate the magnitude of the expected response.

Where therapeutic interventions or new procedures are planned in-vivo, small-scale pilot studies will be first established in 2-4 mice prior to full experiments to assess variability.

Although we seek to use three models of arthritis in this project, where possible, we will prioritise two of them:

The Monoarthritis Model where only one joint in the limb is affected by the disease, meaning the other joints in the limb can be used as a control, reducing the need to assign control animals.

The Serum Transfer Induced Arthritis Model where all four limbs are affected meaning each animal will generate more data, therefore needing less animals to achieve our scientific aim.

We will stop our experiments at any stage where our findings fail to show any significant increase in our understanding of the mechanism by which the cells and molecules of interest function in inflammatory arthritis. In addition, the literature will be also continually reviewed to ensure that we are not repeating published work.

Breeding colonies will be managed in line with the best practice guidelines. Particular attention will be paid to genetic stability and good breeding performance. Data from breeding animals are readily available from the in-house database and will be used to make decisions on future breeding strategies and to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced. Cryopreservation of colonies not required in the short term will be considered.

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 first is monoarthritis where only a single joint is affected. We induce this model by injecting the mouse subcutaneously with an antigen and then seven days later a further antigen injection into a single knee joint. This model may produce mild lameness and therefore is the most refined for us to be able to study how the joint responds to inflammation.

However, this model does not necessarily reflect the human condition where many joints are affected and therefore, we need to induce the polyarthritis model which involves several joints and more accurately reflects the human pathology. Induction of this model is by one of two methods:

1. Collagen Induced Model (CIA) – where the animal receives a subcutaneous injection of antigen into the lower dorsal area.

2. Serum Transfer Induced Model (STIA) – where the animals receive serum from another strain of mouse that spontaneously develops arthritis via the intraperitoneal or intravenous routes.

Both methods create an acute model of polyarthritis meaning we do not need to keep animals for long periods of time post induction.

These targeted approaches will limit the use of persistent models of joint inflammation (e.g. TNFΔARE) to only those experiments where it is needed to model that specific aspect of the disease.

The maximum time a single mouse is likely to have arthritis is 5 weeks as these models are self- limiting. We may give mice treatments that lessen pain and discomfort that might occur as part of the inflammatory response. This may include pre-emptive treatment with analgesia.

We will also utilise a genetically susceptible mouse model of arthritis (TNFΔARE). This is a poly- arthritis model which is progressive from an early age. To limit the harms to the animals we will use a breeding strategy that does not require the homozygous animal to be produced and we only use heterozygous males for breeding to wild type females. This means the pregnant female does not have an extra burden of arthritis whilst pregnant. Stud males are given a twice weekly therapeutic agent to prevent the onset of disease during their stud career, which has been adapted from treatments used in patients with arthritis (e.g. anti TNF biologic therapies).

We will also be studying the effects of our treatments before inducing either model of arthritis. This could be in adult animals, or we may treat the pregnant female. In the case of pregnant females, the female will not be exposed to the induction of arthritis, but the offspring may undergo arthritis induction at 8 weeks of age to assess the long-term effect of our treatments.

We will also undertake gene mapping to study the origin of the different subsets of fibroblasts and macrophages. Such genes are activated by the single administration of a gene inducing agent which will switch on fluorescent markers in specific cell types.

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

Less sentient animals do not recapitulate aspects of the RA pathogenesis in humans. Adult mice, to be used in this project, are the lowest mammalian model for human skeletal and immune systems in which we can test the impact of gene manipulation and evaluate the efficacy of therapeutic interventions.

We will not be able to use animals that have been terminally anaesthetised as the duration of experimentation (days) exceeds the time that animals can stay under general anaesthesia.

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

All mouse models will run for the shortest possible duration to minimise suffering. We have also made refinements to the housing of the animals to cater for any disability arising from arthritis. These refinements include long drinking spouts on water bottles, soft and warm flooring, non-tangling nesting material, food on the cage floor, use of the most refined route of administration, and hydrating food.

We have refined a few of our protocols over the last decade.

We will use established reagents and protocols that we have developed and refined over the last 5 years to treat the mice. Therefore, we will not need to perform unnecessary toxicity studies and will be able to use the lowest doses of agents that are well tolerated.

We have refined and streamlined as much as possible the models of arthritis that we use.

Importantly we have very clearly defined humane endpoints. We have refined procedures of cellular transfer, including intra articular injection, so the smallest volumes can be injected.

Where necessary, males carrying the mutation will be used for breeding with wild type females as this means the female does not have the burden of the effects of the mutation alongside pregnancy.

Where necessary, male mice, transgenic for the gene of interest will be mated with wild type females in order to exclude indirect effects on the progeny derived from gene over-expression in the pregnant female.

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

To ensure the best use of the most appropriate animal models, our experiments will be carried out according to the LASA guidelines: Laboratory Animal Science Association (https://www.lasa.co.uk/) providing guidance toward animal experimentation and welfare.

When planning for animal experiments, we will check the PREPARE: Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (https://norecopa.no/PREPARE) checklist to ensure an optimal study design and be aware of different elements to consider before and during the study.

Finally, we will follow the ARRIVE guidelines: Animal Research: Reporting of In Vivo Experiments (https://arriveguidelines.org/) for reporting and describing our in-vivo experiments when writing scientific publications to ensure a comprehensive and clear description useful for other research projects using animal models.

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

We will attend local 3Rs events and sign-up to the NC3Rs e-newsletter to stay informed about the latest 3Rs advances and updates.

42. Neural basis of visual perception in the mouse cortex

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Vision, Perception, Cortex, Neural networks, Mice

Animal types	Life stages
Mice	adult, 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?

We aim to understand how the activity of neurons in the cortex leads to visual perception, and specifically:

(1) how cortical neurons integrate movement and semantic information to form a coherent percept of dynamic visual stimuli.

(2) how the underlying cortical networks can distinguish between self-generated dynamic stimuli from externally generated ones.

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 essential processes we investigate in mice are also active in humans, contributing to our understanding of general principle of computation in mammalian brains. Neuronal interactions giving rise to the coherent percept of dynamic stimuli likely underlie other binding processes, as those characterizing a vast spectrum of cognitive processes. Additionally, our methods and observations can be applied to evaluate therapies aimed at restoring vision in individuals who are blind.

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

The primary outcomes consist of scientific papers and conference presentations, aiming to advance our comprehension of brain computational principles. Additionally, we develop methodologies employed in neuroscience studies to manage equipment, collect data, and scrutinize recorded information. Collectively, our research might influence the development of devices designed to emulate brain vision particularly for applications in machine learning and artificial intelligence such as artificial vision in robotic applications or self-driving cars.

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

The primary beneficiary of our research is the scientific community which will find our methodologies and investigative approaches ideal for the study of a great diversity of open questions in systems and networks neuroscience. Our outputs will also attract great interest in industry, particularly in the field of artificial vision, which is a central topic in the development of autonomous agents relying on vision to interact with the environment. Our studies are organized into projects, spanning 1-3 years. We share interim findings at global conferences as projects progress. Results are documented in scientific papers, as well as on preprint servers. Once published, we freely share data and provide access to any newly developed software and methods.

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

We share the output of our work through publications, review articles and conference presentations. We also make available data and software using open-source databases and repositories. We advertise our work in the laboratory website and in linked social media platforms. We will strive to establish collaborations with other laboratories, especially those that are part of broader consortia such as the International Brain Laboratory, which includes 22 laboratories in 6 countries that share resources, experiments, and publications to address cutting-edge scientific questions.

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.

Mice, together with other mammalian species, generally follow a common brain blueprint, prompting neuroscientists to focus on the murine model for the rapid breeding, quick maturation, established husbandry, and genetic manipulation capabilities. This makes mice a resource-efficient and impactful choice for research. We study visual perception in adulthood, separate from early period of cortical plasticity. Henceforth, our procedures are targeted on young adults.

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

Standard breeding procedures (Protocol 1) are employed to produce transgenic mice. Following this, certain mice undergo injections either in the brain or systemically to modify specific neuron types, inducing the expression of particular genes. Subsequently, the mice progress to behavioural and neural measurements (Protocol 2). During a surgical procedure, a small head-post is implanted to allow for stable positioning of the head during behavioural and neural recording experiments.

Simultaneously, a cranial window is typically created to facilitate optical access to the brain. Tracers or genetic material may be injected to label neurons or express genetically encoded sensors or actuators. Although attempts are made to carry out these procedures in a single surgical session, circumstances may necessitate multiple sessions, such as for virus expression or task learning by the mouse.

A subset of mice undergoes training for behavioural tasks involving fluid rewards. For example, head- fixed mice might be trained to manipulate a steering wheel to indicate a stimulus position. Training sessions, lasting 1-3 hours, are conducted daily for 4-12 weeks, followed by weeks of consistent trained behaviour. To manage water intake, we regulate their fluids, supplementing as necessary based on daily weight monitoring, adjusted to maintain a target percentage of their starting weight.

Throughout or around this training period, brain activity may be recorded or manipulated.

Upon completion of a series of experiments, typically spanning 1 to 6 months, exceptionally (<5%) up to 12 months, the mouse is euthanized. Typically, the heart is perfused, and the brain is extracted for anatomical investigations.

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

We anticipate all mice to maintain good well-being without discomfort. For this, every significant procedure is followed by a minimum of seven days of recuperation, and we administer measures for both pre- and post-operative pain relief. Certain substances may induce momentary weight reduction or slight abnormalities in motor function. Additionally, mice subjected to a chronic implant may encounter a mild decline in mobility for 2-3 days until they acclimate to the added weight. This will be aided by welfare checks and supportive recovery. Mice displaying these indicators will undergo vigilant observation. Those undergoing water control typically have a loss of up to 15% from their initial body weight due to reduced consumption. To avert potential adverse effects, we assess and supervise them daily for signs of dehydration. In instances of dehydration or if weight descends below 90% of the initial body weight, we supply additional water and food incentives. We reintegrate mice into the experiment only after they have regained weight.

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: 25% Subthreshold (Protocol 1), 25% Mild (Protocol 1), 50% Moderate (Protocol 2)

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 unravel the connection between brain function and visual perception. To achieve this objective, we must explore the brains of animals engaged in visually guided behaviors. This investigation is not feasible through computer simulations or in vitro preparations as they diverge too much from typical brains, offering limited insights into perceptual mechanisms. Similarly, human studies are impractical due to the inability to measure neuronal activity precisely or at scale. Human brain activity is only observable through coarse methods like MRI or EEG, lacking details about individual neuron activity and the possibility to perturb the activity of neurons with single-cell precision. Similarly, during brain surgeries in human patients, limited recordings are possible, monitoring only a fraction of neurons in tissue prepared for removal, thus providing rather incomplete insights.

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

We use computer models to aid in interpreting data from animals and formulating hypotheses for animal experiments. These models serve as a complement, not a replacement, for animal usage.

Why were they not suitable?

Our limited understanding of brain anatomy and physiology places significant constraints on our capacity to emulate the brain through computer models. Due to the numerous unknowns about the brain, these models, when used in isolation from experimental data, they lack the ability to generate powerful insights into brain functions. Instead, computer models enhance our research through an interplay of experiments and modelling. We favour the use of models based on artificial neural networks, whose computational logic is inspired by the operations observed in brain networks. For example, a computer model can simulate neural data enabling us to scrutinize the presence and significance of specific elements in real neural ensembles, thereby confirming their role in the observed brain functions and enabling the formulation of new experimentally testable hypotheses.

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 approach is based on estimating the success rate of experiments in each research project and relating it to the quantity of mice required for a meaningful statistical sample. For instance, not all implants ensure a valid optical access to brain networks, and not all injections of genetic material can lead to sufficiently good expression of optical indicators or optogenetic constructs. Although we have extensively streamlined these procedures, some degree of intrinsic variability remains. In support of this approach, we have extensively reviewed existing literature to validate the customary number of mice essential for attaining statistically meaningful outcomes in such studies. Generally, we deploy 10- 15 mice per experimental group.

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

For our research, we have pinpointed dependable genetically modified strains and will continuously scrutinize the literature to unearth novel strains that might further enhance the data obtainable from animals. The NC3R's Experimental Design Assistant has been instrumental in guiding our experiment planning. Whenever feasible, individual mice are employed in multiple experiments. For instance, in cases where we aim to record and manipulate activity, the same mice can first be utilized for recording and subsequently for manipulation. In addition, we've pioneered chronic recording techniques that allow us to record from the same mice across various experimental scenarios, thereby substantially enhancing the robustness of our observations while concurrently diminishing the requisite number of 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 objective is to diminish our dependence on triple and double transgenic breeding approaches, characterized by numerous pairings and limited usable output for experiments. Our strategy involves substituting these methodologies, whenever feasible, with the administration of viral vectors through injections, commonly delivered retro-orbitally or in the tail vein.

We also adhere to top-notch procedural recommendations for the breeding in our colony, with a keen focus on genetic stability and reproductive efficiency. Utilizing our detailed database, we strategize breeding choices to sustain a population yielding solely the strictly necessary mice for scientific investigations.

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 combination of wild-type mice, usually of inbred origin, and mice with genetic modifications. The genetically altered strains encompass benign mutations enabling the tagging of specific cells, facilitating the monitoring and manipulation of their functions.

For incentivizing mice to engage in tasks, we implement water control, a method proven to be remarkably efficient while causing minimal distress. Mice generally tolerate water control without experiencing any detrimental effects, and their body weight serves as a reliable indicator of health prior to the onset of dehydration symptoms (such as a hunched posture or piloerection). This approach enables us to adjust individual water intake as needed to avert adverse effects.

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

We want for our findings to be applicable to humans, and the human brain shares a basic blueprint with that of fellow mammals. Consequently, we conduct our research in a mammalian species, the mouse. Achieving our objective of unravelling the connection between brain activity and visual perception necessitates the state of wakefulness and, often, observable visually guided behaviour. As a result, the majority of our recordings involve mice in an awakened state, as opposed to those under terminal anaesthesia.

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

We observe the well-being of our mice on a daily basis. In order to alleviate stress, prior to the experiments, we systematically acclimate the mice to handling and head fixation. Special consideration is given to mice that have undergone surgical procedures and may have particular requirements, such as incorporating supportive palatable food to ensure weight stability and administering medication for pain relief and to facilitate the cleaning and healing of wounds under the guidance of NVS. Mice under water regulation undergo daily weight checks and are examined for indications of dehydration.

Adjustments to their water accessibility are made or they are exempted from water control as deemed appropriate.

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

Best practices for refinement are ensured by observing:

1) Guidance and publications from the NC3Rs and Laboratory Animal Science Association: (https://nc3rs.org.uk/3rs-resources)

2) The ARRIVE guidelines: (https://arriveguidelines.org/)

3) PREPARE guildelines

4) Most recent guidance on refinements to rodent head fixation and fluid/food control for neuroscience; NC3RS. 2022. https://www.sciencedirect.com/science/article/pii/S016502702200231X

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

We regularly follow institutional and national initiatives centered on the principles of replacement, reduction, and refinement (3Rs). This involves attending welfare meetings

and 3Rs events, engaging with the National Centre for the Replacement, Reduction, and Refinement of Animals in Research (NC3R), as well as communicating with the Named Animal Welfare & Care Officer (NACWO), the Named Veterinary Surgeon (NVS) and the Named Information Officer (NIO). Additionally, we stay informed by subscribing to the NC3R's newsletter.

43. Investigating tissue immunity 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

Tissue immunity, Ageing, Autoimmunity, Neurodegeneration, kidney disease

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?

To improve our understanding of how immune cells work in different organs in response to infection and inflammation, and to determine how these tissue responses are influenced by age, sex and the microbiome (the bacteria, viruses and fungi that live in the gut, nose and 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?

The immune system mediates responses to infection and to tissue damage.

Historically, the study of immune responses has focused on organs where immune cells develop or are activated. These are called 'lymphoid organs" and include bone marrow, thymus, spleen and lymph nodes. However, there is an increasing appreciation that some immune cells live in non-lymphoid organs, for example, the kidneys, gut and meninges (the

membranes that line the brain), and these have been largely ignored in immunology research. These tissue immune cells are involved in local responses to injury and infections within organs, but our understanding of their function and interactions, how they change with age is unclear, and will be the focus of this work. We also don't know how immune cells in the gut, or in other organs, are influenced by the microbiome, which can be radically altered by antibiotic treatment, or whether these tissue responses contribute to sex-differences in disease susceptibility is unclear,

This information is important to develop better treatments for diseases that are tissue focused, including in the gut (inflammatory bowel disease), kidneys, joints (rheumatoid arthritis) and brain (neurodegenerative diseases like Parkinson's Disease or dementia). These chronic diseases are currently incurable, affect patients for decades, and have a significant impact on their lives and ability to work, with substantial health-economic implications. For example, dementia affects 1 million people in the UK, and is an ever-increasing problem for an ageing population, affecting 1 in 11 people over 65 years of age. We know that infection and inflammation in peripheral organs such as the gut and kidney can accelerate the progression of the neurodegenerative diseases that cause dementia but we don't know why or how that happens. Our work will shed light on the mechanisms by investigating how immune challenges in one organ affect immune responses in a distant organ, potentially identifying new treatment strategies. Some of these diseases also have a strong bias to one sex. For example, systemic lupus erythematosus (SLE for short) is a disease that leads to inflammation in the kidneys, and is more common in women, with a 9:1 female to male ratio. In contrast, kidney disease caused by high blood pressure is more common in men. Understanding the mechanisms that drive these sex-based differences will help develop more personalised therapies.

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

We anticipate that this project licence will generate important **new knowledge** about tissue immune responses and how they are affected by age and the microbiome, and how they differ between sexes.

We will ensure that the information generated is widely disseminated, and have a strong track record in producing **publications** containing our work, including in the highest impact journals. For example, our previous project licence we produced data that was included or informed more than 40 manuscripts.

We would anticipate a similar publication output.

In addition, we will also produce **'methods' papers or book chapters** to ensure our experience of best practice is available to the field.

Our lab also has expertise in using new technologies that generate information about how the genetic code of cells is translated into action, by measuring genetic messenger molecules called 'RNA'. This information is called 'transcriptomics' and includes tens of thousands of RNA measurements. We will make these data available for future use by researchers in **public repositories**, as well as in **open access browsers** that ensure investigators without computational or analysis skills can use the information, as we have done previously.

We may also file for **patents** where we identify potential new treatments.

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

This project will improve our understanding of tissue immune responses in different parts of the body, of relevance to a number of diseases where the benefits from our research outputs will be realised by **researchers**, **clinicians** and **patients**. This includes diseases in:
The intestine – our work will provide information that can be used to develop treatments for gut inflammation (conditions like inflammatory bowel disease), infection and cancers (long-term benefit).

Kidney and bladder immunity – our studies will help identify strategies for the prevention and treatment for urinary tract infection, kidney injury (autoimmune and sterile) and chronic kidney disease. This will help prevent people from getting kidney failure, with important personal and health economic benefits; dialysis accounts for 2% of total NHS spending. Older people are more susceptible to urinary tract infections, we don't know why. Our studies will help find out if there are specific ways we can improve renal tract immunity in older people. Similarly, bladder infections are much more common in women than in men, affecting 50% of women at some point in their lives. We don't understand why, and work generated by this project will help to address this knowledge gap (short-term benefit).

Musculoskeletal system - Joints are affected by autoimmune inflammation (eg, in rheumatoid arthritis) and by infections and they are also 'barometers' of systemic inflammation – with joint (and muscle) "ache" being common symptoms of systemic viral or bacterial infections. Our work will help delineate the cell type-specific mechanisms contributing to joint defence and inflammation, and sensing of circulating immune stimuli and will help us understand whether joint inflammation can influence immune activation in other organs (short-medium term benefit).
Central nervous system organs – brain and meninges. There is currently limited information about the immune cells in the membranes lining the brain (the meninges). Our work will help determine how these cells defend the brain from infection, for example in diseases like meningitis and encephalitis (short-term benefit). We will also assess how activation of immune cells in peripheral organs like the gut, kidney and lung, can affect meningeal and brain immune cells. We have already found surprising links between B and plasma cells in gut and CNS, but will extend this to include other immune cell types, delineating how this affects neuroinflammation and pathology in neurodegenerative diseases such as Parkinson's disease. This potentially opens the way for treatments that are delivered via the gut to modify CNS immunity, for example as oral vaccines that protect from meningitis (long term benefit).

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

We will maximise our outputs via **research collaborations** and **dissemination of our work**.

Collaboration:
We have a strong track record for collaboration. In our previous project licence and work we participated in a number of collaborative projects with researchers locally, nationally and internationally.

We have on-going collaborations with some of these scientists, and will continue to operate in an open, collaborative manner, disseminating best practice and helping other in the field to maximise research outputs.
Dissemination
We disseminate information in open access papers as well as in methods and protocol

papers, for example, we have previously made tissue dissociation methods available on Protocols.io.

We also use oral and poster presentations at conferences to disseminate our work to experts in the field. On average, our group members give at least one talk/lecture per month, including at international conferences.

In addition, we actively engage the public in our work via engagement with social media platforms and public lectures, ensuring a broad reach of our science.

Where we generate transcriptomic data, this will be made available for future use by researchers in public repositories, as well as in open access browsers.

Species and numbers of animals expected to be used

- Mice: Wild type and genetically modified mice: 18,700.

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 are using mice for our research for several reasons:
Mice are genetically very similar to humans, with around 90% similarity in protein-coding genes, including many immune-related genes, They therefore provide a useful model for a variety of immune and infectious diseases, with many examples of this in the literature of how research in mice has identified important pathways in human disease.

Practically, there are many genetically modified mouse strains that enable the role of different immune cell types to be examined; mice can be genetically modified to remove a gene that is required for a specific cell type to develop, for example, removal of the Rag2 gene generates a mouse which has no B or T cells, two crucial immune cell types. More complicated genetic models are also possible, for example, a gene encoding a signalling molecule can be removed in only one cell type. There are also models that allow the removal or over-expression of genes to be switched on at a specific time or place, using harmless chemicals or light. We can also make mice that have been modified so that they have the same genetic variant that is found in some people with immune diseases. Finally, there are several mouse strains that have fluorescent immune cells, allowing their movement to be investigated in real time within a complex tissue environment.

As well as these different mouse strains, there is also a lot of prior experience and models of infection for the gastrointestinal and renal tract, that can be used in mice.

This includes genetically modified pathogens that can be used to track specific immune cell responses.

Our project will use mice after birth, including aged mice, to investigate changes in immunity with age.
Altogether, these features of our model organism uniquely allow human-relevant immune responses across different tissues to be assessed and compared in a way that would be

difficult in any other model organism.

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

We will use genetically modified animals, for example, mice that are either deficient in, or have too much of, an immune cell type, or and immune signal. Some of these mice will be aged to around 24 months to assess the effects on immune responses to tissue ageing. Most of these genetic modifications do not cause any symptoms in homeostasis (the healthy, unchallenged state).

In some cases we will change the immune system of the mouse by generating "bone marrow chimeras". This is done by giving irradiation to remove the bone marrow immune cells, followed by the introduction of genetically modified donor bone marrow. This allows mice to be generated that lack specific immune cells or immune signalling molecules without making a new mouse strain, avoiding lengthy breeding strategies. Following irradiation, mice may temporarily show reduced appetite and are at increased risk of infection. They will be weighed daily and food sweeteners/supplements offered to increase oral intake if required. They will also be assessed for evidence of infection and treated appropriately if needed.

We will also use genetically modified mouse models of neurodegenerative diseases (eg, Parkinson’s disease) to stimulate inflammation in the central nervous system (the brain and spinal cold). These mice develop disease as they age, and most show no symptoms until they are >14 months of age. At this time they may show reduced mobility and oral intake. In most cases, we will not age them to this point, rather their tissues will be taken much earlier, when disease is evident if you examine the brain under a microscope, but mice do not have any overt symptoms.

We will use models of autoimmunity - these are diseases where the immune system attacks our own cells and tissues, rather than pathogens. There are inbred strains of mice that spontaneously develop autoimmune inflammation in the kidneys as they age, and we will use some of these models.

Mice will be challenged with immune stimuli (subcutaneous (under the skin), intranasal (via the nose), intravenous (into the vein), intra-peritoneal (into the abdominal cavity) or into the bladder via a catheter. These immune stimuli will include microbes, and effects on tissue immune cells will be assessed across different organs. Mice are regularly monitored post-challenge.

Mice will be exposed to gut inflammation (colitis) or infection (such as salmonella) by introducing bacteria or a substance (dextran sodium sulphate (DSS)) that causes inflammation orally in water or into the stomach. Following this, mice may develop weight loss (up to 15% of body weight). They may also develop pain and this will be treated with analgesia as needed.

For all protocols, we may monitor the immune response by taking urine or blood samples. Animals will experience mild and transient discomfort from blood sampling.
Experiments will be performed on young and old animals (including those of 12-24 months of age), and on both males and females.

Most of the challenge models are short (less than 1 week) but sometimes we will induce an immune response in one organ, wait for this to resolve and then re-challenge several weeks later, after recovery, with an infection in another organ.

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

The expected impacts on animals vary according to the protocol.

For protocols involving challenge with pathogen or pathogen-associated molecule, mice may experience weight loss, piloerection (hair stands on end), hunched posture and reduced movement. There is no pain associated with these models. These models are short-lasting, typical duration 1-3 days.

For protocol involving intestinal inflammation or infection, mice experience weight loss, diarrhoea, and blood in their motions, as well as some abdominal pain. They may also become hunched and show reduced movement. The active phase of our models last around 7 days. After this, the infection is cleared (eg, salmonella) or the colitis resolves (DSS-induced colitis).

For protocols involving kidney injury/infection, mice may lose weight and may become hunched and show reduced movement. Kidney diseases are generally not associated with pain as the organ does not contain pain neurons. Typical duration of these experiments is short (1-3 days). We do have a model of chronic pyelonephritis.

In this case there is a repeat infection, but this is cleared spontaneously within 3-5 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)?

Mild 54%
Moderate 46%
Severe 0%

What will happen to animals used in 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 tissue immune cells in different organs respond to immune challenge and infection. Our focus is on gut, kidney, bladder, articular joints, brain and meninges. Each tissue has a different and complex mixture of structural cells and immune cells, and unique environmental cues that are generated by their homeostatic functions, eg, the kidney as some regions which have a high salt concentration. One part of the what we want to understand is how immune cells in these different organs influence each other, including by moving between one organ and

another. All of this complexity means that these processes cannot be recapitulated using cells in a dish (in vitro), and an in vivo (live) model is needed.

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

We have continued to develop a number of assays to replace and reduce animal use, including:
In vitro assays performed on murine cells immediately *ex vivo*, as well as on mouse and human cell lines.

Use of human tissues. We have obtained ethical permission to use human kidneys donated for transplant that cannot be used and optimised protocols to extract tissue-resident immune cells. We have also set up a perfusion rig to interrogate the behaviour of human immune cells in the whole organ (kidney) *ex vivo* following different types of manipulation. This model will allow us to recapitulate some diseases in this ex vivo perfused human kidney and helps to replace and support mouse models of kidney injury.

We have also progressed a new ethics application to use kidney biopsies taken from patients with kidney diseases, which was approved in 2023. This helps to replace the use of disease models in mice.
We also obtain human intestinal and bladder tissue from our local Biorepository that retrieves tissue samples from organ donors.

For brain and meningeal tissues, we are working with an academic neurosurgeon and Neuropathologist to obtain human tissues from operations or post-mortem.

Organoids – this system allows cells from an organ to be cultured in a dish, where they form a 3D structure that is similar to some aspects of how an organ works in real life. It is limited by the fact it usually only contains one of the cell types that are present in a real organ in vivo. We have developed a bladder organoid model and are collaborating with experts who have kidney, brain and intestinal organoid models.

Why were they not suitable?

The assays and tissue sources that we use for *in vitro* and *ex vivo* studies are useful, but they cannot completely re-create the complex tissue environment and many different cell types and cell interactions that occur *in vivo*. This is particularly important for our project, as in many cases we are trying to assess how an immune stimulus or infection in one organ in the periphery, for example, infection in the gut or kidney, affects immune cells in the central nervous system. These questions simply cannot be addressed without an *in vivo* animal model. Furthermore, when considering how tissue immune responses are influenced by ageing or the microbiome, *in vitro* cell stimulation or organoid assays are limited in what we can specifically assess. For example, ‘ageing’ a cell line or organoid in vitro cannot recapitulate the many complex signals tissue cells would receive *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 estimated number of animals are informed by the Home Office annual returns of procedure data from our current project licence. We will have similar numbers of researchers working on these projects and are likely to have a similar number of cre-flox complex crosses. We also have a similar number of protocols, and the majority are identical to our previous licence. Therefore, estimates based on our previous animal use should be reasonably accurate.

With regards to how the estimates of animal numbers were historically generated for specific experiments, we have support from the lab bioinformatician who has expertise in statistics and helped us with calculations using the observed endpoint variations in historical experiments in the lab, or from published studies, to calculate the minimum mouse number that can be used whilst ensuring that the results are statistically significant. For most protocols, our previous work has determined the difference in mean, the standard deviation of the experiments to achieve 5% significance level and therefore how many animals per observation are required to detect a significant difference.

We also use control animals/groups for all experiments (appropriately age and sex matched, ideally cohoused in order to minimise intragroup variability due to differences in microbiome. Where possible, we use blinding at all stages of our experimentation and analysis.

For situations where we are using a challenge or model that we have not used previously, and for which there is no published literature, we will use small pilot studies to ensure there is no excess harm to animals.

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

We will minimise the number of animals used by carefully powering studies so that we use the minimum number of animals required to show a statistically significant effect. Power calculations and experimental design will be informed by the NC3Rs' experimental design guidance and experimental design assistant, utilising additional local statistical advice and support for randomisation and blinding where needed.

Where randomised animal groups are needed, we will use appropriate methods, such as the Random Function in Excel.

We will also endeavour to minimise the introduction of systematic variations by ensuring that there are lab standard operating procedures (SOPs) for sample collection, and that there is in-house training for the specifics of tissue collection to ensure consistent fixation, quality of dissection, to minimise post- mortem delay and ensure proper storage conditions according to the experimental end-point assay.

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

To optimise animal use, we will encourage tissue sharing. Within the group there is a tissue sharing scheme where all naïve wild type animals and most experimental animals are offered to other lab members, in case that organs that are not the main focus of the study can be utilised. Where possible we also collect additional tissues for storage

(fixing/freezing) for future use, with documentation of stored tissues enabling other group members to utilise for method optimisation and training studies.

We also try and maximise the use of animals that are surplus to requirements for the original experiment by making them available to other researchers to address other biological questions. In particular stock, we have emailed the establishment 3R emailing list and shared these animals.

We have also provided cells from genetically modified animals for in vitro work to other researchers.

Our licence also includes the ability to perform non-terminal imaging to allow the same mouse to tracked repeatedly rather than requiring multiple mice at different 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.

The organ infection, inflammation and autoimmune models in our licence have been chosen because they are relevant to the human diseases we are investigating. In all cases, we use the protocol with the least severity to investigate the hypothesis under question. For immunisation and pathogen challenge models, we use the lowest dose possible that can generate the immune response under investigation. Similarly, we will use the shortest duration possible, determined by which part of the immune system is being investigated. This means that when assessing innate immune responses, very short experiments (sometimes even lasting only 4 hours) can be used. The challenge models will use a route of administration relevant to human vaccination, therapy or disease, for example, intravesical (into the bladder) delivery of bacteria using a urinary catheter to recapitulate human urinary tract infections, or skin injections, analogous to human vaccination. We also routinely provide environmental enrichment for animals and wherever possible house animals in groups to minimise distress.

To avoid breeding new knockout strains, we will use bone marrow chimeras. To create chimeric mice, irradiation is used to deplete the host cells before introducing donor cells which are then expanded within the host. This allows reconstitution of mice in such a way as to investigate a specific immune cell type and avoids the need to generate new mouse models or perform complex crosses which require many mice.

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

Our project is focused on studying immunity in organs such as the kidney, gut, joints, bladder, meninges and brain. We will use post-natal mice, including aged mice. We cannot use mouse embryos as their immune system and organs are immature. This means that immune cells don't necessarily respond to immune challenges in the way that adult

animals would. The structural cues present in organs are also different in embryos, for example, there is no high sodium environment in the kidney.

Non-mammalian animals are limited because their organs are very different structurally, for example, they may not even have synovial articular joints. Their immune cells are more rudimentary and have different receptors and responses to mammalian immune cells, meaning that they are not a good model for human organs or diseases.

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

We employ general measures to ensure mice are less stressed, including enriched environments, and housing mice in social groups. Where orally substances are given, we make sure they are as palatable as possible, using flavoured jelly, paste or milk shake liquid.

To ensure we minimise the severity and duration of protocols, we use frequent monitoring of relevant clinical parameters and endpoints, for example weight, piloerection, hunched posture, protein on urine dipstick. This ensures humane end-points are adhered to. We have score sheets that are specific for different protocols, for example, for gut inflammation and infection models, these sheets include documentation of weight, diarrhoea severity and whether there is blood in the stools. For kidney models, we use relevant biomarkers, for example blood urea or creatinine and urine protein, which can be monitored longitudinal, ensuring the shortest duration of experiments.

There are also specific protocols where we can minimise the distress to animals by combining procedures. For example, when the administration of more than one substance is required at the same time-point and using the same route, they will be combined in one procedure.

When generating bone marrow chimeras, mice may temporarily show reduced appetite and are at increased risk of infection following irradiation. In the intestinal infection/inflammation models, mice also may lose weight. In these protocols, mice are weighed daily, but we also modify their monitoring to increase the frequency if weight loss if more accelerated that initially appreciated. In addition, sweeteners are offered to increase oral intake.

We will also use genetically modified mouse models of neurodegenerative diseases (eg, Parkinson’s disease). These mice develop motor symptoms when they are old (>14 months of age), but in most of our experiments, we will not age them to this point, rather tissues will be taken much earlier, when disease is evident microscopically in the brain, but there are no overt symptoms that would distress the animals.

We are continually assessing how the procedures can be refined in order to minimise the discomfort that the animals may experience. Members of the lab holding personal licences who undertake procedures under the previous project licence meet regularly to review operational procedures. This has resulted in the implementation of changes that, for example, reduce the procedure time and hence the period of anaesthesia without compromising performance.

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

Will use the published guidelines to inform experiments, such as the ARRIVE and PREPARE guidelines: http://journals.sagepub.com/doi/full/10.1177/0023677217724823 As well as guidance from the Laboratory Animal Science Association, (LASA) https://www.lasa.co.uk/current_publications/

We will also follow the guidance available via NC3Rs publications and newsletters.

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

We are signed up to the local NC3R emailing list. We will also regularly check information on NC3Rs website. We receive regular newsletter and bulletins from our Named Information Officer and obtain the latest practical guidance from Laboratory Animal Science Association (LASA), Institute of Animal Technology (IAT), and the Royal Society for the Prevention of Cruelty to Animals (RSPCA) to ensure we have up to date information about recommendations and advances in animal techniques.

We also regularly attends immunology meetings and conferences, including in neuroimmunology (eg, Keystone Neuroimmunology) , and these include presentations of new methodology and will adopt any new techniques that refine our experimental methods.

44. Immune function in the extended tumour microenvironment

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

Cancer, Tumour microenvironment, Immune function, targeted therapies - immunotherapies

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 aim to use mouse models to dissect the mechanisms of immune response and dysfunction during tumour development to design alternative therapeutic approaches.

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?

According to the CRUK statistics, there are 375,000 new cancer diagnosis in the UK every year. Half of these patients die within 10 years of diagnosis even after several rounds of treatment, costing the NHS considerable resources, such as human, money, and time.

Incidence of cancer is on the rise according to statistics, with breast at the top of the yearly diagnosed cancers. While more research is needed to understand the biology of the different cancer types, we also require identifying and test additional treatment options to

enable patients to fully recover from the disease. It is also relevant to test new treatments with low incidence of side effects to improve quality of life, without the risk of diminishing treatment efficacy.

It is understood that for cancers to develop and progress they need to shut down the body's immune defences. The development of immunotherapies, targeting the activation of specific immune components (such as T-cells), have been a breakthrough in anti-cancer treatments. Although remarkable effects can be produced in patients, even full tumour regression, only between 20 and 50% of patients benefit from these therapies, with high degree of side-effects. These drawbacks are the result of partial immune cell activation but also due to gaps in our understanding of how the immune system interacts with the tumour and its surroundings (the microenvironment).

Here we propose to study the extended microenvironment, with emphasis on the lymph node, to understand how to improve patient outcomes. We will use current immunotherapeutic approaches, but also will be testing promising new co-treatment strategies.

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

Our studies will enable us to understand the biological and molecular mechanisms of immune dysfunction during tumour progression that can then be exploited therapeutically.

We expect to publish at least two peer-reviewed experimental articles deriving from these studies (one with results relating to B-cell specific dysfunction within the lymph nodes, and one for mechanistic response after immunotherapeutic interventions).

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

In the short term (2 years), these studies will provide insights into the molecular cascades responsible for the tumour immune response, during tumour development and immunotherapeutic interventions, which will be published in peer-reviewed journals.

Hence, beneficiaries of this projects will be other scientists and clinicians working in this field.

In the long term (3-10 years), we hope that the primary beneficiaries of these studies will be patients undergoing anti-tumour treatment with immunotherapies. Our ultimate goal is to improve the life expectancy and the quality of life of patients.

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

We aim at publishing into open access journals and will make the publications freely accessible. We will also link possible genomic data to appropriate servers. This will maximise the reach of our discoveries in the scientific community.

We plan to present and discuss the data at different meetings, e.g. the European Association for Cancer Research congress, the British Society for Immunology Congress, among others.

We plan to use these data to strengthen our collaborative network, which include leading clinical centres in different European countries such as Germany, Italy and the UK. Results will be discussed with these collaborators, and where appropriate we will take steps

towards clinical testing and applications.

Species and numbers of animals expected to be used

- Mice: 1320 – 2 years (2970 – 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 need a model with a complex, functional immune system as is found in humans, and which is lacking in lower species. Thus, mice represent the least complex system that possesses the parameters needed to yield meaningful data translatable to human disease. Cancer models based on cell lines, and/or co-cultures, are very limited in the reproducibility of clinical disease. Although they can be used as pilot studies, they only address partially the complexity of cancer development and cell-to- cell interactions.

In our studies we will use murine-derived cancer cells and implant them into immunocompetent mice. This will enable us to fully understand the anti-tumour immune response during tumour development and treatment. Immunodeficient mice can be used to validate specific immune cell contributions. We will use adult mice, since melanoma and breast cancer, our initial models of choice, preferentially develop during adulthood in humans.

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

Animals will be injected with tumour cells (sub-cutaneous injection for the melanoma model, orthotopically within the mammary fat pad for the breast model) and monitored for health and tumour growth.

Animals will then be randomised to receive different treatments, typically via intraperitoneal or intra- venous injections. Animals will be maintained for up to 3-4 weeks or up to 12 weeks (depending on the study), to monitor tumour progression and treatment response, and then they will be humanely killed.

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

Previous experience with these models has shown that the mice tolerate well these tumours, with minimal adverse effects. The tumours only grow locally (no metastasis are detected within the duration of these studies), in the injection site. Their size and location does not result in pain or discomfort or interfere with bodily functions. On occasion, there might be mild weight loss, mild piloerection, mild hunched appearance and, in rare cases, ulceration of tumours. Some of these effects (e.g., ulceration) constitute a humane endpoint and others are expected to be short-lived (from a few hours to possibly 1-2 days, depending on the sign).

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 be using mice and the maximum expected severity is Moderate.

However, we expect only 30% of mice to reach the moderate severity, and 70% to experience a mild severity.

What will happen to animals used in 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?

Tumours are complex systems which contain not only tumour cells but also fibroblasts, blood endothelial cells, lymphatic endothelial cells, pericytes, adipocytes, smooth muscle cells, fibroblast, and immune cell populations all of which interact, adapting as a tumour develops and evolves. The current in vitro models cannot accommodate the numerous interactions required to mimic tumour development and the complexity of the immune response.

In vitro multi-cellular/component models are used to identify and validate responses identified in both murine and human cancers. Their use allows the definition of direct interactions between different cell populations (simplified model), test potential responses to therapy before these can be fully explored using mice, and test possible dose-range responses. They are also critical for us to home in on specific mechanisms following data generated in mice.

However, the complexity of complete tissues and a changing tumour environment cannot be fully recreated in a plastic culture vessel. To do this we require a living system with aspects of the immune system comparable with humans. Such a system does not currently exist in non-animal alternatives.

Mouse models of cancer development produce similar responses to human tumours and continue being good models to identify immune function and test therapeutic agents as well as their influence in immune function.

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

We have considered relevant websites such as NC3R and FRAME, to identify alternatives to the use of animals in this specific research project. Complex multi-cellular in vitro tri-dimensional approaches can be used for pilot studies but lack the complexity of chemical signals required to completely address the questions related to immune-tumour interactions, immune-stroma interactions during tumour development, that will be addressed in this project. Nevertheless, we will use ex-vivo cell culture of immune cells, in combination with fibroblast and/or tumour cell lines, or their derived metabolites, as pilot

studies to minimise the use of animals. These cultures will be performed in either 2D or 3D cultures. Our lab has also used 3D thick slicing of lymph nodes These studies will also enable validation of biological signal cascades. They will also enable us to perform initial testing of potential anti-cancer agents to use in combination to current treatment strategies, to determine their activity and toxicity, and test end-point treatment outcomes.

Why were they not suitable?

As mentioned before, the complexity of the tumour microenvironment cannot be fully replicated in vitro, rendering only partial analysis of the different components. The tri-dimensional in vitro models are also not reliable predictors of response to therapy since we are not able to replicate the systemic influence of the 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?

We have calculated that we will need approximately 12-15 animals per treatment group. This estimate is based on a sample size estimation conducted in collaboration with colleagues with bio-statistical expertise, and using knowledge from previous experiments with similar experimental outputs and our own experience.

We will be using 2 models, 4 groups (treatment naïve, tumour group and 2 treatment groups) per model. Each experiment per model, will require a total maximum number of 66 mice. Treatment groups, without tumour, will also be included for the analysis of unspecific treatment effects. We expect to run these as pilot studies with a small number of animals (6 per experiment, 3 per treatment).

We anticipate running up to 20 sets of experiments during the 2 years of the project (45 sets of experiments accounting for 2970 mice in a 5-year period), testing different combinations of treatments and collecting the appropriate research outcomes. When different therapies will be tested at the same time, we will endeavour to use one control group (vehicle control) for multiple treatments.

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

We have conducted sample size estimations, which are based on our previous experience in animal research and have used the NC3Rs EDA. I have also consulted our institutional biostatistician.

We extensively use complex in vitro systems to generate data without animals, but these are still not able to recreate a living animal. We will specially use in vitro strategies to conduct preliminary studies for the screening and selection of potential compounds to test in our in vivo treatment strategies. Each compound will be tested against a panel of normal (such as fibroblasts and immune cells) and tumour (such as B16F10 melanoma and

E0771 breast cancer) cell lines, as single or multi-cellular cultures.

Our main goal is to determine which compounds can directly, and indirectly, promote activation of different immune cell components. These will also help us determine concentrations to use during in vivo studies.

From each mouse we will collect both the tumour and associated tissues i.e. lymph nodes that are analysed using multiple methods (such as qPCR, imaging, flow cytometry, ELISA). We will also extract tissues from the mice to further use the in vitro systems described above, which complement and help refine our research design, following ARRIVE and PREPARE guidelines. We will also archive tissues for posterior analysis and validation purposes.

These approaches allow us to gain as much information from each animal as possible and reduce the numbers we need to use.

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 technical and biological replicates are collected, bolstering the reproducibility of data obtained, we will collate data from several experimental repeats. Mouse groups will be randomised, and experiments will be conducted in a blinded fashion where possible, with reference to treatment vs. control groups. In these cases, for example, staff would be blinded to solutions they administer to not bias tumour measurements. Most studies have known safe dose for drug use. In vitro titration will be performed to new drugs before animal testing. For drugs with limited information about dosing in mice, we will carry out pilot studies.

Data and protocols generated by this project will be publicly available. This will reduce the need to repeat experiments in the future.

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 predominantly immunocompetent mice where we will induce tumour development, via orthotopic implantation of tumour cells in two well-validated tumour models. These models use murine cancer cells that only grow locally, in the subcutaneous space, in a controlled manner. These tumours do not interfere with normal behaviour and do not normally result in pain or discomfort of the host.

Treatments will be performed using routes and volumes within current guidelines.
We will always try using the least invasive route of administration of the possible options and for the shortest time necessary to establish the desired effect/s. Testing of these

agents will initially be performed using in vitro cultures, with immune, normal and tumour cells, to minimise side-effects in our experimental animals, and to test new compounds and their dosage.

Our experimental endpoints have been chosen to occur before a humane endpoint (such as ulceration or significant weight loss) is reached.

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

Less sentient species do not possess the same complex immune system as humans or mice, making it hard to extrapolate human responses to tumour development and immune responses. There are several similarities between mouse and human immune system (such as cooperation between the innate and adaptive immune system, the different immune cells present and their responses, maturation and differentiation of the different immune cells) as well as similar circulation system (blood and lymphatic), which are not conserved in other (less sentient) animal models employed in biological research (e.g. C. elegans and Drosophila melanogaster).

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

For the past 10 years I have used the models described in this licence and have continually reviewed our experimental design and approach to minimize adverse effects and maximize welfare, improve husbandry and reduce numbers of animals required. Our previous experience has enabled us to refine the tumour models that we employ by reducing the duration that these models run to gain useful information. As such, our experimental endpoints predominantly occur before a humane endpoint (such as ulceration or any significant weight loss) is reached. Similarly, our work is focused on two well-validated cancer models that rely on localised (subcutaneous), tumour growth with no metastasis and no/minimal adverse effects.

Consistent with the best practice principles, we monitor animals regularly, performing daily checks and tumour measurements. We endeavour to use the least invasive route/s of substance administration within the constraints of each study and try to limit the number of administrations. Where possible, we aim to habituate animals to procedures to reduce stress and liaise with animal care staff in terms of providing treats and positive reinforcement techniques.

Animals will be housed in social environments with enrichment.

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

Sampling and dosing volumes and frequencies will be taken in accordance with local guidelines and will also follow those provided in the Workman et.al. Cancer Guidelines (link below) and good practice guidelines for administration of substances from the NC3Rs.

Daily mouse appearance, activity, posture and other signs (including ulceration) will be scored, as local practices.

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

We will keep up to date by reading scientific articles, attending conferences and animal welfare meetings, internally or organised by external bodies such as the IAT (Institute of Animal Technology) or the NC3Rs. I will also regularly consult the Named Veterinary Surgeon, Named Animal Care and Welfare Officer, Named Information Officer and our institutional 3Rs Champion to ensure animal welfare is maximised and/or explore further potential non-animal alternatives.

45. Blood sampling for whole genome sequencing of wildlife species in the UK

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Whole-genome Sequencing, Diversity, Blood Sampling, Genetics, Conservation

Animal types	Life stages
Wild birds (less than 10 g in body weight) (21 species)	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 collect blood samples from wild bird species in the UK for whole genome sequencing.

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 genome contains vital information about the history of life and the code for the functioning parts of cells and organismal systems. This project will contribute to global efforts to provide open genomic data for a large number of eukaryotic species.

A comprehensive digital library of eukaryote genomes will revolutionise our understanding of life on Earth, providing benefits for biodiversity conservation and human societies. Specifically, this will provide a tool for the addressing of fundamental questions in ecology and evolution, including gaining a more complete understanding of processes that are particularly relevant in an era of global change, such as speciation, adaptation, and coevolutionary relationships and interactions in both natural and human-dominated ecosystems.

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

The project will provide blood samples from British wildlife for whole genome sequencing.

The processing of these samples will contribute to the continuous development of new and refined methods for the sequencing, storage, and use of genomic information. The data produced will be openly available for the global community to utilise in constructing future scientific and conservation endeavours.

Importantly, we foresee that the public availability of reference genomes for multiple wildlife species will act as a pre-requisite for analysis in many projects involving biodiversity genomics. This would reduce the necessity of capturing wildlife species for DNA collection in the future and save researchers the time it would take to sequence the genome instead of answering their research question.

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

- By this end of this project, researchers from the UK and abroad will have free access to high- quality genomic data (i.e. reference genomes) of all wildlife species included in this project.
- Conservationists will have access to key genomic data to enable relevant research and conservation actions in threatened British wildlife.
- Additionally, other areas of research that are likely to benefit from the genomic data made public during this project include evolutionary biology, comparative genomics, physiological genomics, and genomic medicine.

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

In collaboration with key UK research institutes, whole genome data will be published as open data on platforms such as Genomes on a Tree (GoaT), Sanger Darwin Tree of Life Portal and Ensembl. Finally, they will be submitted to the publicly accessible European Nucleotide Archive database and a Genome Note is published as an open peer-reviewed paper, announcing the new assembly and how it was processed. The information will be collaborated with The Earth BioGenome Project which is part of a worldwide effort to produce reference genomes for all eukaryote species – hence the results of this project being widely available and promoted for use. Protocols developed for sampling and in the lab are also publicly available online, including any new methods, research and development. Any bi- product data or sample material will be stored for use in future research.

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 will sample wild birds, focusing on healthy adults, from which it is more likely to safely

obtain the volume of blood necessary for genomic analyses. Juvenile birds (>50% adult bodyweight) will be considered for sampling if the opportunity arises and the required amount of blood can be taken safely. This would be done in preference to catching another adult specifically for blood sampling especially in the case of rarer species. Hatchlings and small juveniles (<50% adult bodyweight) will not be sampled to reduce the probability of stress-related adverse effects from handling and blood sampling. We need to capture and sample wild animals because for this project we require blood samples from a range of wildlife species generally not kept in captivity.

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

The birds will be captured using standard methods, health checked and ringed by a trained, competent person holding a ringing permit issued by the British Trust for Ornithology (BTO). This procedure will be carried out by trained BTO staff or volunteers as part of their regular bird ringing activities. Animals will be captured using either mist nets or bird traps, which are regularly used by BTO to capture wild birds and have a <1% associated mortality (BTO, personal comments). Immediately after this procedure is completed, captured birds will be blood sampled by one of the personal licence holders covered under this project licence following standard veterinary methods.

If the opportunity to take blood from an individual held in captivity at a zoological collection or wildlife rehabilitation facility, then this may be done in preference to catching from the wild. This may be necessary for rarer species. In these events birds will be handled and caught by trained keepers and/or veterinary staff.

Blood sampling will be performed following guidance from the N3CRs, Guidelines to the Use of Wild Birds in Research (British Ornithological Council) and relevant literature on blood sampling methods in birds (e.g. Owen 2011, Low 2012). From each individual bird, we will collect no more than 10% of the circulating blood volume (calculated as 1% of the bodyweight). Blood draw will be typically conducted using a needle and syringe or by piercing the vein and collecting via capillary action, depending on the size of the bird, typically sampling from the brachial (wing) or jugular veins.

After blood sampling, birds will be released back into the wild at the exact site of capture provided haemostasis is confirmed and the animal is fit for release. Birds will be held captive for a period typically no longer than 30 minutes from capture until release, and handling will typically last for 10 minutes or less. As all birds will be ringed for individual identification, we have a high level of confidence that blood sampling will not be repeated in the same individual.

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

We expect transient stress and mild pain associated with handling and venepuncture following good veterinary practice, from which the birds are expected to recover within 5 minutes of being released. Typically, we do not expect lasting effects on the animals associated with the regulated procedures used in this project.

Rare adverse effects can include injury during capture (<0.5%; mainly slight abrasions) and mild post sampling haemorrhage (<1%). Any adverse effects will be monitored by a trained individual licence holder by carefully observing the animal from capture until release. If any adverse effects are detected, animals will be further monitored in a quiet, darkened area protected from the elements, and treated as necessary. This can include

ensuring haemostasis in the rare case of haemorrhage, by placing firm pressure on the wound and administration of oral fluids.

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 95% of the animals will undergo mild procedures, specifically these animals are likely to experience short-term, mild pain as a result of capture, handling, and blood sampling using a hypodermic needle following good veterinary practice.

We expect that less than 5% of the animals are likely to experience short-term moderate pain or suffering as a result of rare adverse effects as injury or haemorrhage. If this occurs, and the animal does not recover after 3 hours of adequate treatment in the field, the animal will be euthanised using a Schedule 1 procedure (e.g. dislocation of the neck). This ensures pain of any significant intensity is of no more than a few hours duration and not severe.

What will happen to animals used in 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 need to obtain DNA for whole-genome sequencing. Unfortunately, with the current technology, the quality and concentration of DNA that is required for this analysis cannot be obtained in wild birds using non-invasive sampling methods such as environmental DNA, faeces, saliva, or feathers.

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

Currently, there are no non-animal alternatives to obtain the required amount of DNA necessary for whole-genome sequencing. The current laboratory methods recommend at least 0.2ml of fresh whole blood to maximise chances of high-quality DNA extraction, hence we will aim to collect 0.3ml where possible (DToL Tissue and Blood Sampling Standard Operating Procedure: Chordata: Vertebrata: Aves).

Post-mortem tissue samples from freshly deceased animals (e.g. from wildlife hospitals and rescue services) will be taken opportunistically whenever possible, thus eliminating the need to capture animals from those species from the wild.

Why were they not suitable?

Non-invasive sampling (faeces, feathers, hair, saliva, eDNA) typically contains low amounts of poor- quality DNA, and is often contaminated with external or foreign DNA and

so is not a reliable or efficient method of collecting DNA for high quality, whole-genome sequencing (Theissinger at al. 2023).

Post-mortem sampling is only opportunistic, and we cannot predict the number and identity of species from which we will be able to obtain post-mortem fresh samples.

However, we will ensure to collaborate with all the relevant facilities to increase the coverage of species sampled following this alternative method. Facilities include wildlife rehabilitation centres, pest control units, veterinary practices and captive breeding populations that may have access to freshly dead specimens. We are currently in the processes of compiling a list of contacts to establish relationships with to facilitate a timely retrieval of post-mortem samples.

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?

According to the BTO, there are 634 wild bird species present in the UK, including vagrants and migrants. We aim to collect DNA from 513 of these species.

Endangered species (i.e., species listed in Annex A of Council Regulation 338/97) will not be used. The number of individuals per species required to be sampled is calculated to ensure no long-lasting adverse effects of blood sampling; i.e., we will never collect more than 10% of the circulating blood volume, derived as 1% of the body weight of the bird. We will sample the fewest possible individuals per species to adhere to this rule, up to a maximum of 5 individuals. To estimate the number of individuals that will be sampled for this project, the species are classified into four weight categories. Body weight data for each species were extracted from the literature, and if the body weight was not found, it was extrapolated from similar species. For species in the category "less than 10 g in body weight," we will sample up to five individuals per species. For species in the category "from 10 g to 20 g in body weight," we will sample up to four individuals per species. For species in the category "from 21 g to 40 g in body weight," we will sample up to two individuals per species. For species in the category "more than 40 g in body weight," we will sample one individual per species.

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

Whole, nucleated blood is the most reliable way to obtain whole genome sequencing from live birds, therefore performing this procedure once on one animal is more efficient than potentially just as stressful, non-invasive sampling from multiple individuals. It is the method that is most likely to use the least individuals. To minimise the number of individuals used in this project, whenever possible we will collect the necessary minimum volume from a single individual per species, provided their body weight permits it (as defined in the previous question). Finally, we will make all necessary attempts to include as many samples as possible from post-mortem opportunistic sampling.

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 state-of-the-art molecular methods for whole-genome sequencing, thereby reducing the number of animals required. We will maintain communication with the lab to ensure we are collecting the most functional samples for their methods and keep up with any research and development projects to maximise outputs.

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 targeted species are a large number of wild birds present in the UK, as our project will contribute to building a comprehensive open library of reference genomes for all living eukaryotic species in Britain and Ireland.

Capture and handling will be performed always by trained BTO staff or volunteers following BTO guidelines, which ensure high standards of animal welfare. Other sources of animals may include opportunistic sampling from individuals held at wildlife rehabilitation centres or zoological collections depending on species availability. This reduces the need for further capture and sampling of animals out in the wild. Animals in zoological collections or wildlife hospitals will be handled by trained keepers or veterinary staff.

To ensure minimum levels of pain, suffering, distress, or lasting harm associated to blood sampling, this procedure will be always carried out by a trained, competent personal license holder. Blood sampling method will be based on the least stressful and safest method for the species/individual animal in question.

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

Because we need to obtain DNA from as many wild bird species as possible in the UK in order to contribute to building a comprehensive open library of reference genomes for all living eukaryotic species in Britain and Ireland.

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

Animals will be monitored pre and post sampling and released back into the wild at site of capture following a health check. First aid will be on hand for birds experiencing adverse effects of sampling procedure for example heat source, water source and critical care oral fluids if necessary.
The most welfare friendly method of blood draw will be selected for the animal depending on its weight, biology and vessel accessibility. This will initially be based by reviewing the

literature for blood sampling of wild birds and then adapted and refined with experience.

If any method proves to be unsuccessful or exceeds the number of expected side effects then efforts to mitigate this will be taken such as reviewing the literature again, taking further training or applying alternative blood draw methods. For example, favouring brachial venepuncture over jugular venepuncture if hematoma formation is more frequent than expected in jugular venepuncture.

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

Ornithological Council Guidelines on using wild birds in research. Guidelines September 2023 (birdnet.org)
Darwin Tree of Life Sampling Code of Practice. Template-DToL-Sampling-Code-Of-Practice.pdf (darwintreeoflife.org)

BTO Ringer's Manual.
BSAVA Manual of Wildlife Casualties.
Relevant scientific publications indexed in the Web of Science or Scopus.

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

We will stay up to date on any advances in blood sampling techniques and the handling of wild birds to minimise distress during sampling. This includes reading updates from N3CR's website, British Trust for Ornithology publications, and other relevant material.

We will maintain regular communication with the NVS, NACWO, and NTCO to ensure we are up to date with regulations and appropriate training for all personal licence holders covered by this project.

We will stay up to date on any methodological innovations made at the laboratory processing the samples to always collect the minimum amount of blood required for whole-genome sequencing. If the minimum amount of blood is reduced, this means a lower number of individuals might be required from small passerines.

46. Brain and behavioural phenotyping of genetic mouse models for neurodevelopmental and neuropsychiatric disorders

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Early life, Home Cage, Neurodevelopment, Social interactions, Mouse Behaviour

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?

To use passive monitoring (home-cage analysis) and existing tests to investigate the brain and behavioural characteristics of mouse models for neuropsychiatric disorders, and understand how these change from birth through to adulthood, and into old 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?

Childhood shapes the future adult life. From conception through adolescence and to early adulthood, key developmental milestones are influenced by a wide range of factors; some are inherited, while others stem from physical and/or social environmental elements. For neuropsychiatric disorders it is well established that early life is critical, as three quarters of mental health problems emerge before the age of 24. Yet major clinical and scientific challenges remain: to determine exactly when brain development is perturbed to alter its normal or regular state early in life, why it can manifest into such debilitating behavioural

outcomes, and how modelling of the underlying pathological mechanisms can be harnessed therapeutically. This project will address these important challenges by using Genetically altered (GA) mouse model, where a change has been made to the DNA to alter the function of a gene, to fully characterise these critical time windows.

GA mice are currently in widespread use in biological science and have been shown to be of great value in explaining and understanding the function of genes (the basic physical and functional units made up of DNA, that pass information from one generation to the next) and the ways in which the genes interact with other molecules in the body to express their functions, in a wide variety of biological, physiological and pathological processes. Although the genome (the complete set of genes or genetic material present in a cell or organism) has been sequenced, and >200 gene variants linked to neuropsychiatric disorders such as schizophrenia, the function of many these genes (estimated at approximately half) are poorly understood.

First, we will establish new, integrated approaches for studying the early postnatal period (just after birth). The key to this will be linking the emergence of changes in behaviour in early life with changes in brain development and connectivity, and with adult behaviour. The methods of conducting behavioural genetics have changed very little over the past 40 years, in that they rely on test batteries (or a group of tests), that aim to link the outcome from a standard out-of-cage test to a behavioural characteristic or trait (phenotype), and are generally not applicable to studying behavioural development before adulthood. In recent years, the advances in technology have made it possible to build upon established tests that study the effect on the behaviour response of animals to specific situations, to develop more automated, long-term behavioural testing platforms. These tests enhance and add to the value to the data generated by each individual test not lasting longer than a few hours, thereby building a comprehensive insight into the effects of gene mutations on animal brain and behavioural development over days and weeks instead of looking at smaller ‘snap-shots’ in time.

We will then apply this novel holistic platform to the study of new genetic mouse models that are relevant to human disease and are therefore more "translatable".

We will be guided by the latest genomic discoveries made by our clinical partners.

Our first aim is to identify early changes in brain and behaviour that occur before adult abnormalities in these models. This will allow us to define novel, critical intervention points for neuropsychiatric disorders during early life. We will then test the clinical relevance of these early intervention points by rescuing our engineered mutations using genetic techniques and therapeutics. This will validate these intervention points and provide clinicians with the ability to improve clinical outcomes.

This project aims to improve the translatability of mouse models for neuropsychiatric and neurodevelopmental disease by integrating multiple, longitudinal experimental measures of early-life in individual animals, while identifying time-critical interventions for therapeutic benefit.

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

The main outputs will be extensive novel behavioural datasets, many of which will be included in scientific research publications, and which will ultimately be made available for the research community to re-use as necessary.

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

The main beneficiaries of this work will be the academic research community, particularly those involved in understanding the contribution of genomic variants to neuropsychiatric disorders. However, it is hoped that this research will have translatable benefits (such as the guidance of best practice and/or development of therapeutics). We envisage that our findings and datasets will be of interest to industry partners looking to develop and/or refine novel therapeutics for neuropsychiatric illness.

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

The data will be retained as a permanent digital record of the tests. The initial aim will be to share this data through seeking collaborations with expert scientists in the field of research who specialise in a very specific area in that field of research and can provide expert advice on the work we are doing.

These could be experts in the field of mouse behaviours to medicine and computer data scientists. Ultimately, we hope that much of the data will be made available for others to re-analyse and use. The data will be disseminated through preprint servers, publications, and presentations at appropriate forums.

We aim that our behavioural pipeline will become part of the tests used for preclinical studies more widely, and provide robust, relevant and comprehensive data for screening new therapeutics.

Species and numbers of animals expected to be used

- Mice: 10400

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 main objective of this project is to investigate the brain and behavioural characteristics of mouse models for neuropsychiatric disorders. It is necessary to use mice for this project because ultimately behaviour arises as a consequence of co-ordinated activity within the brain, and the interaction with the internal and external environment. Therefore non-animal alternatives are not appropriate and/or available.

Moreover, we are studying behavioural development, over the full course of the life where possible, across newborn, juvenile and different adult stages. For instance, social phenotypes (social phenotypes are characteristics that relate specifically to interactions with other animals) may be manifested from birth and/or change as the animals age. By including home cage analysis we will refine the study of social behaviour, in order to be able to tease out early phenotypes without interfering interactions of individuals within a group, such as the litter or the another mouse co-housed in the home cage.

Finally, mice are the best experimental model for this project since their physiology and genomics are well studied, and they are a well-established genetically modifiable model.
Typically, what will be done to an animal used in your project?

Approximately 80% mice will undergo a combination of phenotyping tests to understand the contribution of genomic variants to brain and behaviour. Each experiment will use a combination of tests over the life-span of the animals, most of which are non-invasive. A small number will involve general anaesthesia and surgery, such as the insertion of wireless EEG/EMG transponders to take readings automatically and from a distance.

Typically, mice are then terminally anaesthetised and blood and tissue samples taken. Most experiments are expected to end by 15 months of age, with the majority of mice being culled by 52 weeks of age. A minority of experiments, focused on the ageing, will last until 24 months of age.

For some tests it may be necessary to singly house and or have restricted access to food. The aim of the current project is to refine such the set of conditions under which an experiment is conducted so that learning and memory tests may be conducted under reduced access and timed feeding routines instead of restricted access that may last up to 18 hours a day. As well as modifying assays such that they may be conducted under group housed conditions in the familiar environment of the home cage.

Finally, in order to explore underlying changes in brain chemistry some animals may also be given injections of neuroactive drugs.

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

Genetic alterations in the mice used in this project may lead to the development of neurological or behavioural phenotypes. It is expected that mice modelling neuropsychiatric conditions may have delay and/or difficulty in developing skills relating to or involving the process of thinking and reasoning, communication and social interactions with other mice, and these may have some adverse effects such as aggression, memory and sensory input such as the sense of smell, sight and hearing.

However, we do not expect gross changes in basic motor function and behaviours, such as feeding.

Mice undergoing restricted feeding routines in order to use a food-reward for motivation will have reduced access to food. In mice undergoing cognitive testing this may reduce their body-weight to ~85% of their free-feeding weight. Studies have shown that in C57BL/6 mice food restriction increases survival. Thus we do not anticipate adverse welfare outcomes associated with food restriction. To improve the validity of our results we will monitor long-term effects of food restriction by assessment of blood glucose before and after food restriction and in some case body mass composition.

For some tests mice will need to undergo anaesthesia (e.g. for the implantation of devices for remote monitoring). All anaesthetic use in mice carries a risk of mortality and a risk of pain (short-term and long-term), which may differ between genetically altered lines.

In order to explore underlying changes in brain chemistry, some animals may also be given injections of neuroactive drugs. We have extensive experience in giving drugs to rodents using systemic routes and do not anticipate any specific adverse effects. Very rarely animals may display signs of ill-health, and the effects may be exacerbated in new strains brought onto the licence.

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 (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 (wild types and heterozygous animals) will also be born from these crosses, so approximately only 25% of the mice may suffer a moderate severity.

Mice on the behavioural phenotyping protocol are all expected to reach a moderate severity. This is partly due to the phenotype of the mice, in which the genetic alteration could lead to a moderate severity in around 50% of the phenotyping cohort mice (the other 50% being unaffected controls). However, all mice will reach a moderate severity because of a subset of the phenotyping tests causing moderate suffering, for example food restriction for the purpose of motivation in food-rewarded tasks. Additionally, the combined effect of repeating mild tests over the life span of the animal, in order to understand the impact of genomic variants on brain and behavioural development, will lead to an overall moderate experience. This cumulative effect of repeating mild-phenotyping tests may also interact with genetic alterations used in this project to model neurological and metabolic conditions.

Whilst these moderate affects will be short-lasting they will increase the maximum severity of all animals on this protocol to moderate.

What will happen to animals used in 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?

It is necessary to perform whole animal studies to achieve the experimental aims, since in all instances integrated physiological systems are affected. Behaviour is an emergent property of brain function, involving co-ordinated activity both within and external to the central nervous system. Consequently, changes in gene expression and/or gene-product function may affect brain development, and hence behaviour, at multiple levels.

In particular, we are investigating how early postnatal behavioural development changes and leads to adult cognitive deficits. Whilst some preliminary work could and should be done in cells, or using computer modelling, or in other species such as drosophila or zebrafish, it is not possible to replicate this early life environment using cell lines or any other in vitro system.

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

Some preliminary analyses of the effects of our genetic variants on neuronal function will be performed *in vitro* using neuronal cultures. For instance we are examining the electrical activity of neurons derived from induced Pluriopotent Stem Cells (hiPSCs) that carry the equivalent gene mutations seen in human psychiatric conditions, and which have also been engineered in our mouse models. In future we may investigate the effect of neuroactive drugs on modifying the electrical activity of these neuron cultures before testing the same drugs in our mouse models.

With appropriate input and expert advice this approach may extended to using 3D *in vitro* neuronal culture systems, such as brain organoid or assembloid models.

Why were they not suitable?

The aim of this project is to examine how brain and behaviour develops in mouse models of neurodevelopmental and neuropsychiatric disorders using refined home-cage analyses. There are circumstances when earlier work will be done in non-animal alternatives, such as basic characterisation of neuron activity, which will be initially assessed in cellular models. However, the whole organism will be needed to examine how brain and behavioural development change over the life course. The complex nature of how this development interacts with processes such as parental care, nursing and the social environment, also means that non-mammalian species such as Drosophila or zebrafish are not suitable.

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?

Current samples sizes are estimates based on previous data. As we are refining procedures it is difficult to be certain of the correct sample size, however previous data from tests is used to enter into statistical equations that help calculate how many animals are needed in an experiment) for refined protocols to give us sample sizes needed for individual tests. As we are using recently developed test protocols, as a first pass a highly specialised set of calculations that helps scientists decide how many animals would be needed for an experiment, where no previous data is available (G power calculation) may be used to determine sample sizes needed to detect a desired effect size. Where there is no previous data for power equations, resource equations may also be used.

An example of a typical study would be, 24-32 mice per genotype (12-16 males, 12-16 females) will be required for each experiment; most experiments will have two genotypes although some, such as conditional GA experiments may have additional control groups. We anticipate undertaking approximately 15 such experiments per year for every year of this project.

At least 2-rounds of breeding will be required to generate the mice required for the experiments outlined. Thus, in total we estimate we will need to breed 4000 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?

Power equation calculations based on previous data will be used for group size calculations for the phenotyping experiments. Animals will be randomly assigned to experimental groups where possible and experimenters will be blind to groups (e.g. genotype, drug treatment) where possible.

The combination of tests in each experiment will be designed to gather the most meaningful data in order to validate the refinement. Tests which can inform each other will be carried out on the same mouse to remove inter-animal variability (this is the differences between individual mice that can influence the results) and increase the power, thereby decreasing the overall sample size and the scientific utility of generated data. We will also phenotype over the life-span of the animal to get better data from a smaller number of mice and to prove that the new/refined set of experimental plans is as accurate or better at detecting the same experimental outcome as the test it replaces over the progression of disease.

Standard Operating Procedures (SOPs) have been written and used routinely for standard tests and will be written and version controlled for the developing and refined tests. Each iteration of the test in development will be linked to the appropriate version in order to keep track of the assay development and validation in order to inform future refinements. This standardises the way the data is 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?

Efficient breeding will be used to minimise the number of mice being produced for these studies. Genetically modified lines that have already been studied will be sourced from repositories (these are carefully managed stores where the germplasm of GA mice is stored for future use), to avoid remaking of lines whenever possible.

Animals from the colony and tissue/samples will be shared with other researchers with overlapping interests. Pilot studies will be undertaken to generate means and standard deviations for work using back-ground strains for which data is not available. Tissues sampled from the animals used in this project will be shared with other researchers and the data produced linked to that generated by the project, to maximise long-term utility.

In addition, the data generated as part of this project, particularly home cage analysis video data, could be reinterrogated if new analysis methodologies come online during the lifetime of this project.

Alternatively, as we build normative datasets, these could be reused. For example, the use of computer modelling to predict patterns within big data this is especially relevant to time dependent pattern of behaviours, where it is not necessary to generate new data for understanding what is 'the normal pattern' but compare new data generated in GA models to historic data to tease out 'anomalies' that may be indicative of a phenotype.

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 overall objective of this project is to greater understand the mechanisms by which genomic variants contribute to neuropsychiatric illness. To do this we will be examining brain and behavioural development across the lifespan in Genetically altered (GA) mouse models. Mice are chosen as an experimental model since their physiology is well studied and they are genetically modifiable, plus, like people, their brain and behavioural development also depends on the interaction with the social environment (parenting, cage-mats etc).

Many of the behavioural methods used in this project are passive measures (home cage analysis) or measures of spontaneous natural behaviours in response to a stimulus. Of those where training and learning is required we only use appetitively reward tasks.

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

This project is focused on understanding behavioural development in genetic mouse models for neuropsychiatric disorders. Whilst discrete aspects of function can be investigated using in vitro studies, the development of behaviour over time cannot be modelled in a cell culture system. In addition, brain and behavioural development involves complex co-ordinated interaction both within, and external (i.e. with mother and/or conspecifics) to the central nervous system, which are difficult to properly model in non-mammalian species.

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, using tunnel handling where possible, and provided the time to get used to testing rooms and apparatus.

Using passive monitoring using homecage analysis (HCA) from birth to early adulthood and then coupling this with measures of cognition later in life means we can examine behavioural development and adult cognition in the same animal. This longitudinal approach will generate a statistically robust dataset and provide a better understanding of how gene variants associated with neuropsychiatric illness contribute to brain and behavioural changes. Using HCA will also reduce handling and user interactions with animals.

We will only be using appetitively rewarded tasks - for instance using food as a reward (as opposed to aversive learning, such as fear conditioning) - to examine aspects of cognition.

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 establishment has full AAALAC and ISO9001-2015 accreditation. To conform with these standards, we must work to 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
PREPARE planning and ARRIVE reporting 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?

In addition to local advice from the AWERB and NACWOs, we will also regularly engage with NC3Rs via the Regional Programme Manager for our region.
In addition, members of the team at the establishment will attend specific conferences that focus on all aspects of the neurological and metabolic disease, from humans, to model organisms to *in-vitro* (experiments done outside the animal e.g. cell lines) and *in-silico* (computer modelling work such as artificial intelligence and computer learning). Any new developments which could impact these studies will be discussed with the teams at the establishment as well as experts in the field who may advise on this project.

47. Developing the next generation of biologics and gene therapy approaches

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

Gene therapy, Monogenetic disorders, Cancer, Advanced therapies

Animal types	Life stages
Mice	pregnant, adult, 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?

To assess the safety and efficacy of biologics and gene therapy technologies for the treatment of a range of blood, liver and other human 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?

This project entails a combination of basic and translational research to develop safe and efficient biologics or gene therapy strategies for the treatment of blood, liver or other human disorders. These treatment approaches will likely fulfil unmet treatment needs for a range of disorders (e.g. patients with bleeding disorders, mucopolysaccharidosis or cancers).

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

This project will result in new data on the use of biologics and viral vectors for monogenetic and acquired disorders. Based on previous outcomes it is expected that the project will generate new data to enable lead candidates to progress to phase I/II clinical trials, publications, and patent applications.

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

It is anticipated that knowledge from this project will expand the patient population that is eligible for viral vectors as well as the possibility of a repeated dose which could potentially allow lifelong expression of the therapeutic protein from childhood to old age. It is also expected that the knowledge gained in this project will allow us to leverage our current knowledge and expertise into other monogenetic disorders with suboptimal treatment. In addition, this project will allow further development of biologics with improved efficacy and reduced off-target effects.

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

Our laboratory has a long-standing record of collaboration with international groups covering several different areas of expertise. In addition, we have collaborated with industry to translate advances in gene therapy and biologics to marketable therapies. We will continue these relationships to maximise the progress on new targets/improved therapies towards clinical trials.

Species and numbers of animals expected to be used

- Mice: 4200

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, both wild-type stains as well as genetically modified strains. Mice used in this study may either be modified to model the human disease or immune-compromised strains to allow the use of human cells as part of the model.

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

Experimental animals will typically receive a viral vector by a single injection into the tail vein. Mice will then be monitored with routine blood sampling to monitor expression up to 1 year of age.

For studies which are evaluating biologicals for cancers mice will be dosed initially with the the biologic expressed from a vial vector by a tail vein injection. Following stable expression of the biologic (as measured by blood sampling) tumour cells will be implanted with an injection into an appropriate site. Once the tumour is detectable mice will receive injections of human T-cells twice per week. Tumour growth will be measured by imaging and calliper measurements, for up to 4 weeks.

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

project?

For most animals treated with viral vectors we would not anticipate any adverse effect on the animal other than transient distress during the administration of the vectors. In studies in which genetically disease altered models are used the administration of the viral vector would be expected to improve the health of the animal. In cancer studies mice may respond with weight loss following T-cell administration as the T-cells kill the cancer cells, this is an anticipated adverse effect and will be closely monitored as well as the general condition of the 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)?

The majority of the mice are expected to be in the mild-moderate category. 20% mild for breeding and moderate 80% for the studies assessing viral vectors and novel biologics.

What will happen to animals used in 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?

Murine models of human disease serve as an important tool for establishing pre-clinical proof of concepts, and for assessing the efficacy and safety profile and immunological consequences of our gene therapy approaches. The body’s response to gene therapy vectors involves, multiple systems, organs and cell types.

Additionally, the complexity of the immune response cannot be assessed in in vitro settings. The use of in vivo models is essential to refine existing and develop immunotherapies as they allow us to detect in vivo homing and persistence of transferred cells, their efficacy in modifying/preventing disease and off target toxicities, In vitro systems, while useful, do not fully replicate the complexity of immune reaction or disease pathogenesis in vivo and it is essential to use appropriate and robust animal models to understand these processes.

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

We use in vitro studies to gain as much information as possible prior to In vivo studies. For example in designing new gene therapy vectors we will characterise our candidates in vitro using liver cell lines to determine the lead candidates which can then be validated in mice. We constantly exploring new in vitro models, but have not found any that can replace animals.

Why were they not suitable?

In vitro studies cannot capture various aspect that are important for preclinical development of gene therapy products such as long-term expression kinetics of the vector over weeks- months, distribution of viral vectors in other tissues, off target expression, and immune response to the viral capsid and transgene.

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 estimated from grant applications and based on previous studies that we have carried out with appropriate effect sizes and statistical significance.

We have estimated the number of studies per year that we will carry out, but could be lower if we are able to rely more on in vitro studies.

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

We will use tools such as the Experimental Design assistant with data generated from previous experiments to carry out in vivo studies with the minimal number of animals required to reach a statistical conclusion to the research question.

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 non-invasive imaging and serial blood samples to maximise the information obtained per animal with longitudinal follow-up of the same animal over a period of time.

We will use efficient breeding strategies and will store frozen embryos to avoid maintenance of a colony not actively required.

Pilot studies will be carried out if we are unable to estimate from past experience or from the literature the number of animals required for an experiment. For example, we may need to carry out a pilot study to establish the dose of viral vectors required to achieve expression in a therapeutic range.

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 our work on gene therapy for monogenetic disorders, we will typically evaluate viral vectors in wildtype mice. Mice are administered an IV injection of a virus and then followed for weeks or months with serial blood sampling to monitor expression and immunology. We have used this model repeatedly and have found to date that all transgenes are well tolerated without any impact to the health or wellbeing of the animal. In studies in which a mouse KO model is used, administration will either partially or fully restore the deficiency and the mice will benefit from improved health compared to untreated littermates. For our oncology studies, we will use immunocompromised mice as is standard for the field to allow engraftment of the human tumour cells. Mice will be implanted with tumours to either grow as a localised solid tumour, or in a diffuse manner if administered by IP or IV injection.

These models are well characterised by us and widely reported in the literature to allow close monitoring of the mice.

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

Genetic modification of mice is well-defined and their immune system has been intensively studied and bears extensive similarities to that of humans.

The majority of reagents and tools required for the proposed plan of work have been designed for use in mouse models and for establishing safety and efficacy. To our knowledge, no other species of lesser sentience can fulfil the requirements of this programme to the same extent as the laboratory mouse.

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

The proposed mouse models are already well established in our previous projects, All the protocols have clearly defined humane endpoints and the majority of mice will be humanely killed with an appropriate schedule 1 method within 12 months of receiving a gene therapy vector, or gene-modified immune cells, or earlier, whenever endpoints are reached. All experimental procedures are carefully monitored by experienced staff within the research group and in the animal facility. Animals exhibiting any unexpected harmful clinical signs will be killed using a humane method.

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

We will keep up to date with the NC3Rs ARRIVE guidelines.

Guidelines for the welfare and use of animals in cancer research.

Workman et al British Journal of Cancer, 2010 (and revision when published).

Administering substances: Refining procedures for the administration of substances.

Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. British Veterinary Association Animal Welfare Foundation/Fund for the Replacement of

Animals in Medical Experiments/Royal Society for the Prevention of Cruelty to

Animals/Universities Federation for Animal Welfare. Morton et al Lab Animals, 2001.

Taking blood samples: NC3R guidance, available on website.

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

We will maintain awareness of the NCR3s guidance and will adopt these should relevant guidance change. We will do this by regular review of guidance on the NC3Rs website and subscription to the monthly newsletter. We will attend the University's annual conference and any relevant training. A member of the research group working under the PPL will attend the local user group meeting. We will also keep an open dialogue with the NACWOs and NVS.

48. Enhanced Bone Ingrowth into Orthopaedic Reconstruction devices

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

Orthopaedics, Bone Ingrowth, Osseointegration, Medical Devices

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?

This project will enable orthopaedic medical device companies to study bone ingrowth into novel porous bone contacting surfaces intended for use in orthopaedic reconstruction devices such as hip and knee replacements.

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?

Joint replacements have dramatically improved the quality of life for millions of people by removing the pain associated with degenerative joint disease and by restoring motion. Traditionally, artificial joints were anchored in place using a cement to hold the artificial joint to the bone in which it was implanted. However, problems associated with cements have led to the development of cementless implants.

Such implants rely on bone to grow into a porous surface on the artificial joint to provide anchorage.

Novel structures and materials are being developed to enable more bone to grow into these porous surfaces which will improve implant fixation, reduce implant loosening and will ultimately lead to longer lasting orthopaedic implants and fewer early revision surgeries.

It is therefore essential to have robust in vivo models, predictive of the environment in which new implants will be used, in which the degree of bone ingrowth and implant fixation can be assessed.

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

The products being developed and evaluated are intended to promote greater bone ingrowth compared with existing orthopaedic reconstruction devices. This will improve implant fixation and therefore improve patient's lives. It is expected that the data from successful studies will support regulatory approval and launch of new products.

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

This is a service licence which will enable orthopaedic medical device companies to access expertise and models that have been developed and validated over the last two decades in order to evaluate new products.

It is anticipated that members of the human population requiring knee or hip replacements will benefit from the work conducted under the authority of this licence.

These patients' everyday activities will have likely been impacted by degenerative joint disease leading to the requirement of a replacement joint. If the long term fixation of available joint replacements is improved they will last longer than current products which will improve patient's lives.

It is also anticipated that the surgical implantation of novel structures and materials is simplified so orthopaedic surgeons will also benefit.

These benefits will in turn reduce the cost burden on healthcare providers.

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

The offering of validated bone ingrowth models as a service means that numerous medical device companies will be able to evaluate their products in these models.

Where confidentiality is not breached data will be shared across organisations and where possible, publications of the work conducted under this licence will be considered.

Species and numbers of animals expected to be used

- Sheep: 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.

The adult sheep is our large animal model of choice for implantation studies designed to assess implants of a size representative of the clinical situation. The hind limbs of adult sheep contain sufficient cancellous (spongy) bone to allow the assessment of bone ingrowth into an implant of a size that is suitable for subsequent mechanical testing and histological analysis.

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

Sheep will be acclimatised to the facility and handling procedures prior to use.

On the day of surgery they will receive a pre-medication containing an analgesic (painkiller) and will then be anaesthetised for the surgical procedure. The surgical procedure will be performed aseptically (in a sterile manner that is free from harmful bacteria and microorganisms) and will involve the implantation of small coupons of material into the cancellous bone in the hind limbs. Surgical sites will be closed and the sheep will be recovered from the anaesthetic.

A typical procedure will take approximately 1 to 1.5 hours from anaesthetic induction to wound closure.

Post surgery analgesics will be used as required.

Following recovery images such as x-rays may be taken to assess bone ingrowth into the implants no less than 2 weeks apart.

At the end of the procedure sheep will be humanely euthanised and the implant/host tissue construct will be removed for testing and analysis.

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

It is expected that there will be a degree of post-operative discomfort and lameness which will be controlled by analgesics. This isn't expected to last longer than 24-72 hrs following surgery.

Sheep will be single housed during the immediate post-operative period. This is to prevent injury before the sheep have fully recovered but as they are a herding animal this could cause some distress. To minimise this distress a line of site will be provided to adjacent pen mates and group housing will normally be re-introduced 24-72 hrs following surgery. Re-introduction to group housing is expected to be without incident.

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 procedure.

What will happen to animals used in 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?

Bone repair is a complex process involving cellular repair mechanisms and inflammation which cannot be studied using in vitro cell culture studies.

The endpoint measures required to study the amount of bone that has grown into the implants and the strength of repair provided by the implants are both histological and mechanical requiring the use of living tissue.

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

In vitro cell culture studies involving the use of synthetic bone scaffolds either unloaded or under some load to try to emulate the clinical environment in which the final products will be used.

Why were they not suitable?

In vitro cell culture studies are useful as a screening method to assess the effects of novel materials on the viability of cells. These types of studies will be used to screen out any potentially harmful structures or materials before bone ingrowth is studied.

However, for the bone ingrowth studies themselves, animals need to be used to study inflammatory responses and repair 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?

The total number of animals has been estimated based on typical study sizes and expected numbers of studies required for the duration of the project. Power calculations will be used to determine the number of animals required for each study and this will be dependent on the specific study objectives.

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

As this is a service licence the experimental design phase for each required study has not yet happened.

When it does FRAME and NC3Rs guidance will be followed regarding reduction opportunities and the NC3Rs Experimental Design Tool (EDT) will be used where appropriate to inform the design of studies. Statisticians will be consulted in the planning stages of in-vivo studies to determine the appropriate study design, number of groups and number of animals required. Studies will typically be designed to 80% power, although this could differ, and could be designed, for example, as either superiority or non- inferiority studies with appropriate limits depending on specific study objectives.

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

Historical data will be used to power studies where it exists. Otherwise pilot studies will be utilised to inform the design of subsequent pivotal studies. Control items will be used in all studies ideally to provide intra-animal comparisons, where test groups and control groups are implanted in the same animal. Animal variability will be reduced as much as possible by the sourcing of a consistent and reproducible supply of sheep.

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 sheep will be used in this project as they have limbs which are of a sufficient size to study clinically relevant sized implants. They also have a sufficient volume of cancellous bone to ensure that the implants are fully surrounded by bone and normal healing occurs at a rate that is clinically relevant. The models proposed have been developed, validated and refined under four concurrent Project Licences over a period of 20 years.

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

Adult sheep have limbs which are of a sufficient size to study clinically relevant sized implants. Juvenile sheep would have smaller limbs which may not have sufficient cancellous bone to study the required implants. These would also heal much quicker potentially masking any improvements provided by novel implants. Less sentient species have been ruled out due to their size.

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

The models within this licence have been developed, validated and refined over the last two decades however, opportunities for further refinement will always be considered. Guidance from institutes such as NC3Rs will be followed where appropriate.

Acclimatisation periods will be utilised and refinements in post-operative care and pain management will be utilised where these are proven to reduce harms to the animals.

Animals will be group housed where possible and where single housing is required following a surgical procedure, a line of sight to a pen mate will be provided by not having solid pen sides. Group housing will be reintroduced as soon as possible after a surgical procedure which is expected to be without incident. Good ventilation is essential when animals are housed indoors and when possible, animals will be moved out to pasture.

Environmental enrichment methods will be utilised. In sheep these are mainly limited to providing a variety of feed and feeding methods. In addition to feeding good quality hay/haylage ad-lib a scoop of pelleted diet can be added for variety, mineral licks and additional feeds may also be provided and supplements e.g. beet or other appropriate fruit/veg may be fed as a form of environmental enrichment. The method of feeding can also be regularly changed to add variety.

Surgical implantations will be practiced and refined in cadaver tissues as required.

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

LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017), NC3Rs, ARRIVE and PREPARE guidelines will be followed where appropriate.

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

Through general literature review, review of NC3Rs website, dialogue with the Named Information Officer and Named Training and Competency Officer as well as other establishments.

49. Identifying new treatments for obesity and metabolic 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

Gastrointestinal tract, Metabolism, Energy homeostasis, Pancreas, Glucose homeostasis

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

To better understand how the brain and the body communicate to regulate body weight and glucose metabolism, and to identify and investigate targets for the treatment of metabolic 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?

Obesity and its related diseases, particularly type 2 diabetes and non-alcoholic fatty liver disease, are major public health concerns, with a rising prevalence worldwide.
Obesity rates have doubled since 2010 and the World Obesity Federation predicts one

billion people globally, including 1 in 5 women and 1 in 7 men, will be living with obesity by 2030. More than 420 million people worldwide have diabetes, and diabetes causes 1.5 million deaths each year. The consequences of these metabolic disorders are substantial, including increased risk of cardiovascular disease, cancer, liver dysfunction, and premature mortality. These projections highlight the ongoing and increasing public health burden of obesity and related metabolic disorders despite the discovery of better treatments. Understanding how organs including the gut, pancreas, liver and brain interact to control these systems will facilitate the development of novel treatments. This is crucial to improving health outcomes and reducing the economic burden on healthcare systems globally.

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

Studying how the gastrointestinal tract, pancreas, liver, brain and associated systems regulate energy homeostasis and metabolism will give us a greater understanding of how these systems work, how their dysfunction can result in obesity and metabolic disease, and will characterise which processes are involved. This will support the development of novel effective drugs to treat or prevent obesity, diabetes and other metabolic diseases. This new knowledge will be published in peer-reviewed scientific journals, and the most promising data is expected to underpin human studies to further explore the usefulness of specific approaches for treating or preventing disease. In addition, specific approaches used to non-invasively image the gut and other organs are expected to support animal welfare, demonstrating how non-invasive techniques can be used experimentally.

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

Treatment of obesity and the diseases which occur as a result of it, such as diabetes and cancer, costs the NHS more than 10 billion pounds a year. This need is set to overwhelm the clinical capacity of the health service.

In the short term, these outputs will benefit the research group and other researchers in the field, improving their understanding of the systems involved in regulating body weight and blood glucose levels, and identifying promising systems to target with studies that translate these findings into humans. They will also provide useful information relevant to the reduction and refinement of animal studies.

In the medium term, we hope that the results of these studies will inform e.g. dietary advice within the health service, and stimulate drug development programmes within the pharmaceutical industry.

In the long term, the hope is that the translational potential of our findings will be realised, with new dietary or drug treatments resulting in reduced ill health, reduced premature deaths, and increased quality of life for patients at risk of developing or suffering from obesity, diabetes and other metabolic diseases, and a consequent reduction in the wider socioeconomic burden of metabolic disease.

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

The research group is highly collaborative, with links to other groups with complementary expertise in the UK and in other countries. We will leverage these collaborations to robustly test hypotheses and generate high quality data. We also intend to share the knowledge gained through professional societies (such as the Society for Endocrinology) and presenting at national and international conferences and seminars. All data will be

published as soon as possible in open access journals or in established journals where open access can be ensured. This will include negative data to help others avoid repeating such studies, and we will also share negative data though our networks and contacts. Potential new users will be able to find out about our data and identify whether they are suitable for their research purposes through summary information available on our institutional website, and following the presentation and publication of the results of these studies. Appropriate research data will be deposited in and made freely available upon request to academics at higher education and research institutions from an identified community database following publication (e.g. gene expression data in a public functional genomics data repository such as Gene Expression Omnibus).

Species and numbers of animals expected to be used

- Mice: 9895
- Rats: 970

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 as they have biological systems regulating their food intake, body weight and blood glucose very similar to those in humans. They also become obese and develop diabetes, similarly to humans.

We need to use rodents in our project as metabolic disorders such as obesity and type 2 diabetes are complex diseases. The effects of these diseases occur in numerous organs and systems, which can only be studied in whole animals.

Mouse and rat cells are similar to human cells, in the ways they grow, divide, and communicate with each other.

Mice, rats and humans have the same organs and their bodies work in much the same way, and they are also very similar in the way they develop diseases like obesity. They are therefore able to help us understand the systems and mechanisms involved in metabolic disorders and to test potential treatments.

While mice and rats are similar in many ways, in some cases rats are a better model for humans than mice. For example, rats and humans both have an increase in their basal metabolic rate after administration of the pancreatic hormone glucagon, an effect which is not seen in the mouse. In addition, rats are more suitable for certain studies as their metabolic rate more closely matches that of humans, and their larger body size allows for a greater total volume of blood sampling and makes it is easier to visualise certain organs or tissues. However, the mouse will be the default organism to use unless the specific experiment justifies the use of rats.

The work on this licence will use juvenile or adult mice and rats, as they provide the most useful model to understand how the regulatory systems involved in obesity and type 2 diabetes develop and how these diseases manifest in adulthood.

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

Typically, this project will investigate the effects of agents such as nutrients, hormones or other biological signals on the regulation of feeding, body weight and blood glucose levels.

For agents which have beneficial effects on these systems we may explore the mechanisms involved, and whether they are able to improve metabolic disease in rodent models, which will help us to understand whether they might make good targets for human drugs in the future.

Many animals on this project will be used to produce genetically altered models that have specific processes altered, or which have a higher risk of metabolic disease.

Some of these animals will not be used for further experiments, but the majority will.

These subsequently used animals and other animals that have not been genetically altered will undergo different combinations of procedures, depending on the specific question that is being asked. Some genetic models may involve the administration of specific drugs to drive the changes in gene function required, allowing us to control the stage of life when they occur. Other models may use the administration of agents to specifically knock out specific types of cell in a certain region, or may deliver genetic material which allows, for example, the activation or suppression of the activity of specific cells, or helps us to visualise certain cells.

When we want to study disease processes, which may reveal new mechanisms to further investigate as potential therapies, animals may be given diets with altered constituents, particularly high fat diets which will cause them to gain weight. They may have blood samples taken, their response to glucose may be tested by injecting them with glucose and measuring their blood glucose, and they may undergo imaging so we can visualise what is happening to their internal organs non-invasively. Such animals may be kept for five or six months for the disease to completely develop. At the end of the study they will be killed humanely and multiple tissues taken so that we can get the most information from them possible. We will also use models of gut dysfunction, which can reflect the inflammatory changes observed in the gut in obesity and therefore interfere with normal regulation of body weight and glucose. These models will typically involve shorter timelines, of two or three months, and similar sampling and imaging protocols.

Some disease models will be used to investigate the effects of potential treatments.

These animals may also have blood samples taken and be imaged, but they will also be administered a drug, either via pumps implanted under their skin or in the abdomen, via injection or oral administration. To look at the long term effects of these agents, some animals may have as many as seventy such injections or oral administrations.

Administration of these treatments will follow best practice as recommended by Morton et al 2001 (1). Again, samples from animals' organs and tissues will be taken to study after they have been humanely killed. In some studies, animals may be caged alone for up to 28 weeks so that the food intake of an individual animal can be monitored accurately, and to allow us to match the food intake of some animals to a control group, helping us to understand the contribution changes in food intake make to changes in body weight observed.

In other studies, the effects of agents will be tested in healthy animals. We may test the

short-term effects of comparable or related agents administered via the most appropriate route, again according to best practice (1), on food intake or glucose regulation. In some cases we will use drugs to block specific internal processes or genetically altered models with changes to specific pathways to try to determine the mechanisms involved. In such studies, animals may be kept, sometimes singly housed, for four or five months, and the effects of related agents (e.g. amino acids to understand the effects of protein ingestion and digestion) or drugs (e.g. gut hormone analogues with different structural changes made to them), sometimes at different doses, on food intake or glucose homeostasis compared. Some studies will also involve the use of pharmacological agents (e.g. a receptor blocker) to investigate the mechanisms by which an agent drives its effects. To test for possible side effects of the most promising agents we may monitor animal behaviour. We may also use a conditioned taste aversion protocol, which involves water restricting animals to better determine whether they associate administration of a particular substance with an aversive response, suggesting that the substance may therefore be less suitable to develop as a therapy.

A small number of animals will be used for 'tracing' studies, where genetic material is introduced to cells so that these cells and/or cells connected to them can be identified.

One of the approaches we will use is a modified pseudorabies virus, which will be used in short term studies to map the neurons upstream of particular cells, with animals being killed before they suffer any ill effects from the virus.

In a small minority of animals we may also use specific surgery to investigate mechanisms. This might involve the placing of a device that can record physiological parameters in an animal, or it might involve the placing of tubes into blood vessels, the brain or the gut so that agents can be administered. Another surgical model we will use involves transplanting pancreatic cells into the eye of an animal so their development and activity can subsequently be visualised without using invasive techniques. A minority of animals may also be housed in special cages which allow us to monitor their metabolic rate.

A small number of animals may be housed at warmer or colder temperatures than they normally experience so we can gain insight into how they regulate their energy balance under those circumstances.

A small number of animals may also have glucose clamps performed on them (2).

This typically involves inserting tubes into two blood vessels and allowing the animals to recover. Subsequently, animals will have their blood glucose levels kept constant by infusing glucose intravenously while also measuring blood glucose levels, usually in response to the administration of a substance, meaning that the glucose infusion rate required reflects the effects of that substance on glucose metabolism. Such animals will not usually be kept for more than two weeks following tube insertion.

A typical scenario might use group housed animals placed on a high fat diet for sixteen weeks and then singly housed and maintained on high fat diet for a further four weeks.

During this four-week period, animals would receive typically 28 peripheral injections (daily injection) over one month, with food intake and body weight recorded at least three times a week. Animals would undergo imaging on two occasions, normally at the start and end of treatment period, and would have small blood samples taken on the same occasion. Mice would also undergo three glucose tolerance tests, where they would be given an injection

of glucose and five small blood samples would be taken over the next two hours. Overall experiment duration would be twenty weeks.

Morton DB, Jennings M, Buckwell A, Ewbank R, Godfrey C, Holgate B, Inglis I, James R, Page C, Sharman I, Verschoyle R, Westall L, Wilson AB; Joint Working Group on Refinement. Refining procedures for the administration of substances.

Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement.

British Veterinary Association Animal Welfare Foundation/Fund for the Replacement of Animals in Medical Experiments/Royal Society for the Prevention of Cruelty to Animals/Universities Federation for Animal Welfare. Lab Anim. 2001 Jan;35(1):1-41.
Shiota M. Measurement of glucose homeostasis in vivo: combination of tracers and clamp techniques. Methods Mol Biol. 2012;933:229-53.

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

Most genetically altered animals used in our project are expected to show only mild differences compared with unaltered animals. Genetic models of obesity rarely develop complications which cause adverse effects over the time period we plan to study them over. However, additional monitoring will be carried out to make sure that mobility and access to food are not restricted and that they don’t develop other complications. Genetic models of diabetes may cause moderate adverse effects, mainly related to them losing more water in their urine, and we will increase bedding changes and ensure free access to water. These animals can also lose weight due to the loss of glucose in the urine, and will therefore carefully monitor animals’ weight and glucose levels to deal with this. Obese animals may lose <20% of their body weight from their peak obese body weight.

Studies inducing obesity through diet are unlikely to result in adverse events within their proposed duration except for consumption of more water and greater volume of urine production. Typically, the levels of obesity achieved is unlikely to alter behaviour and the induction of glucose intolerance by diet results in a mild condition, with no discomfort to the animals or adverse effects. Other forms of dietary modifications, such as the addition of specific nutrients or tastants (agents which produce a particular taste sensation), may result in reduced appetite, hunger, and weight loss, but are very unlikely to result in weight loss greater than 10%. Animals will be monitored daily and body weight will be measured at least twice a week to ensure they are consuming food and that they don't lose too much weight. Non- obese animals may lose up to 15% of their body weight from their peak body weight.

Obese animals may lose up to 20% of their body weight from their peak obese body weight.

Some of the gut disease models we will use can cause moderate effects, including poor gastrointestinal function leading to diarrhoea and weight loss. A formal scoring system will be used to closely monitor the health of these animals, with those passing prearranged limits being humanely killed to ensure no animal experiences more than mild or moderate disease.

The agents we will study are expected to mostly have been used before in animal studies, within the group and/or in the wider literature. Most of these agents will have relatively short half-lives (i.e. they will only remain present in the body for short periods), mostly less

than 12 hours, and so ill effects will generally only be short-lived. The doses of substances to be given to animals will be carefully considered to limit the size of this effect. For agents we have not used before, we will carefully examine evidence from the literature and will carry out pilot studies with small numbers of animals using conservative doses to limit possible adverse effects. While we will use agents expected to drive body weight loss, we will use doses and protocols which keep such weight loss within specific limits, preventing rapid drops in weight. Therefore, administration of substances should result in no more than mild transient discomfort. Similarly, peripheral blood sampling, imaging, use of metabolic cages and behavioural monitoring should result in no greater than mild transient discomfort.

To study appetite and energy regulation, animals sometimes need to be caged alone to allow accurate measurement of food intake. Singley housing rats and mice can have adverse effects on their behaviour, and animals will only be single housed when required.

When animals are single housed, additional cage enrichment will be provided, and animals will be closely monitored for signs of behavioural changes. Single housing may be required for up to 28 weeks. However, for the majority of animals it will be no more than 20 weeks.

We may study the response to cold exposure of a small minority of animals to understand how this alters their body weight and response to agents. Animals will be carefully acclimatised to any such reduction in temperature, allowing them to adapt to minimise adverse effects.

Surgery may be carried out in a minority of animals to explore specific mechanisms. Such surgeries will be carried out while animals are under anaesthetic. However, these animals will experience some discomfort after surgery and some mild to moderate pain which will be treated with analgesics.

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 levels on the animals in this project licence are:

26% subthreshold
34% mild
40% moderate for both mice and rats

What will happen to animals used in 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 systems regulating metabolism and disrupted in metabolic dysfunction involve complex networks of interacting organ and signalling systems, including the nervous, immune and endocrine (hormonal) systems. These networks are currently impossible to reproduce in tissues or using computer modelling, and thus this project requires the use of animal models. Mice, rats and humans have the same organs, use very similar systems to regulate food intake and blood glucose, and their development of obesity and diabetes occurs in a similar way.

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

We have worked for more than twenty years in the field and are familiar with the non-animal models used to investigate aspects of energy and glucose regulation. We have accessed various databases and guidance to ensure proper alternatives are considered.

We have utilised the EURL ECVAM search guide and The Animal Welfare Information Centre 3Rs Alternatives Literature Search Support guidance to make sure we are using good search practice when looking for alternatives to animal use. We have used databases including ALTBIB and PubMed, and the NC3Rs portfolio and gateway site. We considered the use of cultured cell lines, ex vivo tissue, organoid models and mathematical modelling to address our aims. We also investigated the use of non-protected species and using human data.

When possible and appropriate, substances will be initially characterised using these in vitro and other non-animal methods.

Why were they not suitable?

We intend to use cell lines and tissues to answer specific questions and complement our in vivo studies, as currently occurs in our laboratory. These studies can provide more information on signalling pathways and can be useful in e.g., screening targets for their potential effects on metabolism.

However, they cannot demonstrate the role of specific molecules or pathways in physiology. Eating behaviour can only be assessed in vivo. Glucose regulation is controlled by multiple organ systems and it is not possible to understand the role of particular pathways using isolated organs or cells. Many other metabolic pathways similarly need to be investigated in intact animals because of their complexity.

We have previously carried out mathematical modelling of eating behaviour, which provided useful insight into different aspects of feeding. However, in vivo data was required to generate these models, and they could not provide information on the specific signalling pathways involved, which is crucial to establish potential therapeutic targets. We will thus use modelling where possible to complement and optimise our in vivo studies, but it cannot replace the use of animals.

While non-protected species can offer some insight into energy regulation, their physiology and metabolism is quite different from that of humans. Rodents are extensively used in the study of gastrointestinal and metabolic function, allowing this project to build on existing knowledge. Rats and mice are omnivores, and while there are anatomical differences between the rodent and the human gut, functionally the different gastrointestinal regions work in a similar fashion, and rodent obesity and diabetes models reflect the most important aspects of human disease.

Human data from individual studies and population studies can provide valuable information on the gut and the systems regulating metabolism. However, it is not practical or possible to carry out the mechanistic studies necessary to demonstrate the roles of particular molecules and pathways in humans, thus limiting their ability to identify potential therapeutic targets. We will focus our in vivo studies on pathways most relevant to humans, concentrating on genes and systems in rodents which are known to have equivalents 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?

All animal experiments will be carefully planned. We expect to use significantly more mice than rats, as mice more easily allow the use of genetically altered (transgenic) models; rats will used when required for specific reasons such as aspects of the system being studied being closer to humans than those in mice, or when larger blood samples or larger organs (to facilitate imaging) are needed. It is estimated that approximately 600 standard mice will be used per year, and the remaining number of mice will be transgenic animals, the majority of which will be from breeding programmes to generate mice models of interest. The maintenance of transgenic breeding colonies, the breeding of different lines together, and the need to investigate the effects of specific genes in one particular sex (e.g. only male TallyHo mice, a model of diabetes, actually become diabetic) can necessitate the breeding of relatively large numbers of transgenic animals. Breeding strategies for genetically altered animals will be optimised to minimise the number utilised. We have used our previous experimental data to estimate the number of animals that we will need to use for breeding transgenic models.

Experiments will use appropriate control groups. For substance administration studies this will typically be a vehicle-treated control group. For some transgenic models it will be necessary to compare baseline parameters and the effects of specific agents in multiple genotypes (genetic backgrounds).

For others, we may need to compare the effects of administration of a control gene transfection vector (a tool used to deliver a genetic change in an animal) such as a virus. Such groups will control for the effects of the vector rather than simply for the effects of any necessary surgery. Some studies will not require control groups (e.g. when tracing studies are used to map the neuronal connections of organs or when mice with particular cell types expressing a genetic calcium indicator are used to generate primary culture models or organoids to help us understand intracellular signalling pathways).

Group sizes will be planned in collaboration with statisticians based on expected effects and the variability expected. Tools like the NC3Rs Experimental Design Assistant will also be used.

Statisticians have previously supported us with calculations based on our own pilot data to calculate the minimum numbers of animals required for specific studies to ensure that the

results are statistically significant. For studies where this previous experience is not directly applicable, variability in the primary outcome parameters will be estimated based on past experience and data within the research group, data from the wider literature, and the use of pilot studies for novel approaches. Pilot studies will typically use a single dose of an agent, with dose response effects only being investigated when we have already confirmed the agent has an effect. For specific studies, animals will act as their own controls to reduce the numbers required.

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

We have previously used the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our studies and data analysis. We have used this knowledge in combination with advice from institutional statisticians and other experts where applicable and will again use the EDA to support novel 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?

The literature will be carefully studied to ensure we do not duplicate work already carried out. Where possible, studies will use animals handled by a single person to reduce variation and thus minimise numbers required. Animals will be randomised at the start of individual studies (e.g. by body weight), and investigators blinded to treatment where possible. Breeding strategies for genetically altered animals will be optimised to minimise the number utilised. Pilot studies will be used for novel approaches. Multiple tissues will be taken and multiple parameters measured to maximise the data from each animal. Other tissues will be stored in case they are useful in the future to the group or collaborators. Non-animal studies will be used where appropriate to screen agents to select the most appropriate for in vivo testing. The ARRIVE guidelines will be closely followed for design, analysis and reporting of all in vivo 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 mice and rats in our studies. Mice and rats are the most widely used model organisms for studying metabolism. The systems involved in metabolic regulation are similar in both rodents and humans. The extensive literature available detailing comparable studies in these species will greatly reduce the need for preliminary studies.

The experimental approaches we intend to use have been widely used over the past twenty years, and thus have been previously refined and tested by multiple research teams. In addition, we have direct experience with the majority of these methods, and are familiar with their optimal implementation.

We will use animals given altered diets or administered substances, or with specific genetic changes, to investigate energy regulation, metabolism, and gastrointestinal and metabolic diseases. The transgenic and dietary models we plan to use to alter energy and glucose regulation may result in body weight gain, altered metabolism and high blood glucose levels, but the majority are not expected to cause pain or distress.

We will utilise widely used models of gastrointestinal dysfunction that result in no more than moderate severity. For example, the short-term methotrexate model of small intestinal damage drives minor changes in the gut and animals typically recover within a week. The 'T cell transfer' colitis model, in contrast, results in large intestinal inflammation with features characteristic of human inflammatory bowel disease. These models are well documented, reflect pertinent human diseases with implications for metabolism and produce clinically relevant data, but typically do not progress beyond moderate severity; animals will be carefully monitored to ensure that they do not develop disease of greater severity. Muscle is a major contributor to whole body energy regulation, but to increase or decrease muscle to examine its role, we will use a voluntary running wheel to increase muscle and mild detraining models of physical inactivity (removing the availability of the voluntary running wheel), rather than more severe forced exercise or limb unloading models.

For substance administration studies we will use the mildest administration protocol that will meet the requirements of the study. Narrow gauge needles will be used for substance administration. For chronic studies, where possible we will supplement food with the agent, and where this is not suitable, we will aim to use e.g. minipumps to deliver substances rather than rely on multiple injections. In some acute studies, animals can be trained to consume the required agent in a short period of time, but where this is not possible (e.g. in glucose tolerance tests, when precise timing is vital to determine effects) we will use oral administration or administration by injection.

Where administration is challenging e.g. into specific regions of the brain, we will administer through indwelling tubes implanted under general anaesthesia. For blood sampling studies, the blood volume taken will be the minimum required by the study.

Non-invasive methods will used where possible. Animal suffering will be minimised by appropriate use of anaesthesia and analgesia to control pain. All surgeries will be carried out under general anaesthesia and using appropriate post-operative analgesia. We will frequently discuss best practice with the NVS and NACWO.

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

Human metabolism, and particularly energy and glucose regulation, is a complex system. While the basic action of appetite and metabolic control is a fundamental process for life, its complexity has increased as mammals have evolved. Rodents model human physiology and disease much more accurately than less sentient species. Models such as fruit flies, nematodes or zebrafish have been useful in identifying genes involved in metabolic regulation, but do not reflect the complexity of these metabolic systems and the organ-to-organ communication present in mammals. Mice and rats are therefore the least sentient animals that can be used for this research. These metabolic systems are also different in immature animals, and it is more relevant to human disease to investigate them in juvenile or adult animals with largely or fully developed metabolic and endocrine systems.

Where appropriate we will conduct studies in anaesthetised animals, but this is not feasible for longer term studies, or acute studies where food intake is measured or effects on glucose control are investigated.

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

We will take a number of steps to minimise harm and the chance of adverse effects and suffering in the animals. These include the in vitro investigation of any previously untested substances prior to administration in animals. Animals will also be acclimatised to handling and procedures before any actual experiments are performed. Animals will be monitored frequently, and those subject to novel agents or genetic manipulations will be subject to additional monitoring. Animals will be humanely killed if unexpectedly severe adverse effects are noted. The animals' environments will be routinely enriched with the addition of materials including, but not limited to, nesting material, cardboard and plastic tubes, shelters, chew blocks and sticks and aspen balls. Where animals require single housing to enable e.g. accurate assessment of food intake, additional enrichment will be used Anaesthesia and analgesia will be administered as required and pre-emptively where possible. Experiments will be conducted on terminally anaesthetised animals when appropriate.

As an experienced group we have already refined many aspects of the procedure, such as using narrow gauge needles for substance administration and minimising blood volume required. However, we will frequently discuss best practice with the named veterinary surgeon and NACWO.

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

We will follow Home Office guidelines and regulations and guidance from Morton et al 2001 for dosing and substance administration except for specific noted examples. We will ensure all PIL holders working under this PPL are sufficiently trained and supervised by experienced group members or collaborators before being permitted to work independently. The ARRIVE guidelines will be closely followed for design, analysis and reporting of all in vivo studies. We will also refer to NC3Rs guidelines and website for up-to-date refinements of techniques. Remaining up to date with published literature will also help us to implement any more refined models that are developed.

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

Our institution has a strong commitment to the 3Rs and an active 3Rs programme.

Group members will attend 3Rs related seminars, talks and other events to stay abreast of recent developments in the 3Rs. We will also be advised by local experts regarding the implementation of 3Rs best practice, and will remain up to date with the relevant scientific literature and the latest articles on the NC3Rs website. We will discuss any potential improvements with the NACWO and NVS prior to implementing them to make sure best practice is followed.

50. Neural Circuitry Assembly

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cerebral Cortex, Neural Circuitries, Development, Brain

Animal types	Life stages
Mice	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?

My laboratory aims to understand the cellular and molecular mechanisms controlling the development and maturation of neural 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?

Understanding how neural circuits are formed in mammals is not only crucial for obtaining basic knowledge but also essential for translational research. As there is a growing awareness of the need for translating basic scientific findings to the clinic, it has become increasingly evident that the bridge between basic neuroscience and clinical application must be built on a solid foundation. In other words, it is imperative that we have a better understanding of how the brain works in both healthy and diseased states.

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

At the end of the project, we will be in a position to understand better how mammalian neural circuits are formed and how experience influences these circuitries. 1) we will be

able to decipher why our neurons can learn better during development than in adulthood, 2) the specificity of human synapse development and 3) why cortical interneurons are key for the developing of mature and healthy circuitry.

- We will generate new models to study neurodevelopmental disorders; we will produce open databases with our throughput screenings (e.g. bulk RNA and single-cell sequencing as we previously published on different websites); we will publish our results in a preprint open source version before their publication in any peer-review journal.

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

The outputs of this project will be in the long term.

- Other Research in the field and other fields: for example, our discoveries have already influenced the field of Neurodevelopmental Disorders by discovering a novel mechanism for cognitive deficits in schizophrenia. The impact of cortical interneurons on cognitive function is now recognised in other fields, like Alzheimer's and Depression. Likewise, I would expect that our outcomes on this new project will pave the way to understanding how experience influences the circuitries as they develop and shed some light on future interventions to mimic the state of early circuitries.
- Clinical researchers and clinicians. We collaborate with different members of our institution by cross-linking the main questions in neurodevelopmental disorders so that we can help understand the mechanisms underlying their neuropathology. The Pharmaceutical Industry. The three projects represent a unique opportunity to better understand the mechanisms underlying neurodevelopmental disorders and identify targets for a new generation of drugs. As an example, our translational efforts have already been recognised by Pharmaceutical Companies, which supported part of our research on this topic through their Research Award Program.

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

Based on the work my lab has developed during all these years, I have established a solid ground and recognition for international collaborations and advisory groups, that includes international consortiums and Scientific Advisory Boards. This will allow me to share our work and the newly acquired knowledge, some of which will be published and some unsuccessful; I will continue in this direction. I also plan to disseminate our work at conferences, seminars, pre-print publications, peer-reviewed publications, and social media (e.g., Twitter, now X), as I have done in the past.

Species and numbers of animals expected to be used

- Mice: 20000

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 gold standard for understanding a complex biological system such as the brain since they share 95% of the genes with humans. For example, disruption of human

genes shown to be linked to cognitive disabilities causes similar deficits when altered in mice. Mice are easy to maintain and reproduce quickly and are very amenable to genetic manipulations and analysis. For all these reasons, the mouse is the best species to use in this project.

1. Selection of embryonic manipulation. This is based on technical reasons for gene targeting. For some experiments, the best way to target isolated cells is to manipulate them during early development when they are generated and migrating.
2. Selection of neonatal and postnatal ages. This is our most common choice of life stage based on the development of the circuitries in the mouse cerebral cortex.
3. Selection of adult mice. This is used to study the circuitries in adulthood.

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

Typically, our experiments involve tissue preparation for anatomical analysis or in vitro experiments and injecting substances under general anaesthesia, such as viruses and gene-altering agents postnatally and occasionally at the embryonic stages. The duration of this procedure is 10-15 minutes per animal.

Some of our experiments also involved other surgical procedures under general anaesthesia, such as implants that will be followed by training on sensory discrimination and learning tasks, which typically involve motivation by food reward in food-restricted animals. Recordings and manipulations of the activity in brain regions may accompany this. Other experiments will involve other behaviour tasks with no implantation devices, such as open field, working memory tests or learning tasks motivated by food rewards in food-restricted animals. The duration of the procedure will vary depending on the experiment but typically lasts for 1 month.

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

1. There is a low risk of deaths occurring from anaesthetic or complications during surgery (<1%).
2. Animals are expected to reach moderate levels of severity only during the recovery period immediately following the surgery, which will last around (<2% of the time spent in this protocol).
3. In rare occasions (<1%), adverse effects of surgery may include weight loss of more than 15% of total body weight during a few days, repeated vocalisations, intolerance to handling, persistently altered gait and posture, and excessive loss of coat (e.g. piloerection, unkempt coat).
4. There is also a small probability of infection in the operation site (<1%), indicated by redness, swelling, fluid discharge, intolerance to direct touch or detachment of the implant (<1%).

Some protocols involve motivating the animals to perform the task for food reward by keeping them food restricted at a target body weight lower than their feeding weight.

This task will last for the behaviour training, typically 1 month.

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 under mild severity (about 70% of mice) or non-recovery severity (about 10%). The remaining will be under moderate severity (about 20%). No animals will be under severe severity.

What will happen to animals used in 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?

Understanding how neural circuits are built requires intact biological systems with their complexity. Such complexity cannot be reproduced in computer models or efficiently replicated under in vitro conditions. This is confirmed by the evaluation of other procedures published in www.grame.org.uk: there are no current alternatives to animal experiments for this research.

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

There are two-dimensional (2D) and three-dimensional (3D) cultures (organoids). In 2D cultures, the cells grow as a monolayer in a culture flask or wells attached to a plastic surface. They can be primary cultures, when cells come directly from the tissue, such as neurons from brain samples, or they can be carried on using induced pluripotent stem cells. The 3D allows the cells to interact in three dimensions. They are generated from induced pluripotent stem cells or embryonic stem cells. These cultures provide more in vivo-like cell interactions and morphology, as the 3D shape more accurately mimics a more natural environment and diversity of the cells. They are used to explore different aspects of cell function.

Why were they not suitable?

Although recent advances in stem cell biology have advanced our possibilities to study neurons, we are far from being able to model complex neural networks. 2D culture lacks neuronal diversity, particularly the diversity of interneurons. Although more elaborated, organoids 3D) do not reach the proper maturity to explore the emergence of mature circuitries.

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?

Since the experiments we are carrying out for the next years are very similar to the ones we have been doing in the lab, I calculate the numbers based on my previous Project. I have also considered the percentage of the known anticipated success of surgical procedures, biological variability and measurement variability. For example, experiments on cell-type specific targeting require a combination of overlapping successful injections that the chances of success decrease.

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

During the experimental design, all members of my lab visited the website of http://www.3rs- reduction.co.uk/ for any experimental design. They used the http://www.biomath.info/ website to estimate the sample size. Based on previous experiments in my laboratory, the minimum effect size that can be measured with our biological readout is 20% with a power of 80%, having a high heterogeneity within the experimental group. For example, the sample sizes for synaptic and behavioural analyses are n=4-5 and n=12-15, respectively. However, a power analysis is not always possible, for example, when it is the first time a measurement is taken, since the standard deviation may be unknown. Whenever this happens, we use The Resource Equation.

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

Small pilot experiments are conducted before new experiments to optimise the task's success. We use a factorial experimental design to maximise the data collected from each animal, and breeding strategies optimise using the same mice for different outcomes whenever possible. Mice are randomised in control and experimental groups using a randomisation programme considering age, body weight, and litter.

Following randomisation, each mouse is given a number to conceal their group. Data are analysed without knowing the experimental history to avoid selection bias. We have sought and will seek statistical advice to improve the quality of our design and reduce the number of animals used.

The project has been designed with the goal of reducing the number of animals used. For example, we have developed a database to register every piece of tissue obtained from these animals, which will be efficiently stored for future studies. With more than 30 years of experience using mice as a model system, the experiments are always designed to use the minimum number of animals required to generate statistically significant data in my laboratory.

Since we use cell-type specific targeting of neurons, only a fraction of the mice generated with the breeding will be used for our experiments. However, this will allow us to obtain more accurate results (refinement).

With the explosion of the single-cell RNA sequencing open sources, we can now use some of the already published databases to run bioinformatic analysis and find our genes of interest instead of producing our data. Although this cannot be done for all experimental designs, this reduces part of our use of mice for this specific aim.

In addition, every month, I discuss the management of their colonies with my team

members (one-on- one meetings). Specifically, based on the document prepared by our BSU from the MCMS program, one of my research assistants prepared a document with all mouse colonies (breeding, maintenance, and undergoing experiments) per lab member. Then, the excess number of mice for any of the above categories is discussed and amended if needed. Additionally, I have established a mandatory induction for all new staff that will join my lab to improve the management of the colonies and the experimental design. A senior member of my lab with extended experience in mouse colony management instructs the new staff on the best practices for handling a colony depending on the type of experiments. The use of databases like http://www.nc3rs.org.uk complements this induction.

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 experimental models since they are mammals and share 95% of the genes with humans. Mice are easy to maintain and reproduce quickly and are very amenable to genetic manipulations. In particular, we can use mice to express fluorescent proteins to label the neurons or manipulate the expression of particular genes of interest to investigate how cortical circuitries are built. Then, to investigate the relevance of these manipulations in the function of cortical circuitries and behaviour, we obtain optical recordings and assess behaviour. In the last few years, we have implemented new methods for gene manipulation in a cell-type-specific manner to obtain loss and gain from function experiments. We have generated tools that can be driven by AAV viruses to achieve the function of a given gene simultaneously with the identification of the neuron's morphology. These tools have substantially reduced the need to generate transgenic mice for animal research, reducing the breeding of several generations of mice to obtain the right genotype.

The simultaneous recording of hundreds of neurons also improves the power of our analysis and, therefore, reduces the number of mice needed to complete the experiment.

In addition, we are implementing a new method that will allow us to explore the human neurons in an intact circuit using the xenotransplantation models (transplantation of human neurons into mice). This will also avoid the use of non-human primates.

Most of the methods we will use in this project have been refined in my laboratory over the last 10 years. Maintaining a similar methodology will ensure less animal suffering and distress.

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

Mice share 95% of the genes with humans, and genes linked to disabilities in cognitive function in humans have also been demonstrated to cause similar deficits in mice. Given

our ability to manipulate their genome and their susceptibility to some of the same genetic defects that cause human disease, mice are the gold standard for the type of experiments presented in this Project. Complex CNS behaviour (e.g. EEG, neurophysiological signals related to information processing, social behaviour, learning and memory) can be studied only in intact animals. To understand how cortical circuitries are formed, we must explore the suitable ages for this in rodents. The first peak of synapse formation is at Postnatal day 10-P15, and the full maturation of the circuitry is accomplished around P70-P90, depending on the cortical area. We then need to assess the neural circuitry's function and behaviour when the network is entirely mature. Therefore, younger animals can only be used for this project a few times.

Other vertebrates, like zebrafish, have a forebrain but do not recapitulate the mammalian cortical diversity and therefore cortical function.

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

For new Lab members, rigorous training and reassessments of procedures will be taking place. In all surgeries, we have aimed to reduce the duration time of the procedure at all stages. In addition, we have decreased the delay until the pups are back with the mother after post-operatory in the recovery chamber. To ensure the welfare of the animals, anaesthesia and analgesia will be provided to the mice to avoid any suffering before manipulation or sacrifice for the experimental procedures using approved methods. Currently, we are already using additional local analgesics (e.g. Lidocaine) for surgeries. We are implementing based on the NC3Rs guidance their recommendations for head fixation (https://www.nc3rs.org.uk/3rs-resources/refining-use-head-fixation-and-fluid-control-rodents). The study plans run by the Animal Facility are regularly revised by our Animal Facility managers, who advise on the best procedures to minimise animal harm.

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

- LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery
- The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments)
- NC3Rs-funded video tutorials on the Research Animal Training website (https://researchanimaltraining.com/article-categories/aseptic-technique/)
- Refinements to rodent head fixation and fluid/food control for neuroscience (https://doi.org/10.1016/j.jneumeth.2022.109705)

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

My institution regularly informs me through emails and workshops about the 3Rs updates. We also regularly use the NC3Rs website when we need to design new procedures and update the standard ones. In particular, we follow up closely any updates related to in vivo manipulations of mice.

51. The developmental and physiological role(s) of aryl hydrocarbon interacting protein and its interacting pathways

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

Zebrafish, Development, Metabolism, Pathophysiology, 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?

In humans, loss of function variants in aryl hydrocarbon interacting protein (AIP) cause a severe, multi- system syndrome, characterized by failure to thrive and heart arrythmia that is fatal in human infants. The overarching aim is to understand the role of AIP in development and the cell biological and physiological processes affected by AIP loss of function. Our long term aim is to identify therapeutic interventions to improve outcomes for affected individuals.

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?

Aryl-hydrocarbon interacting protein (AIP) is involved in multiple cellular processes. In humans, heterozygous AIP mutations (mutations that are present in one copy of the gene but the other is wildtype) are associated with pituitary adenoma, giantism and, in studies from our collaborators, autism, but are not life threatening. However, homozygous mutations (mutations that occur in both copies of the gene) lead to death as infants. These infants fail to grow despite direct intravenous feeding at elevated levels. Work to determine the developmental and physiological role of AIP that underlies the disease phenotype is essential to the search for therapeutics.

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

Expected outputs include:

- Establishment of the developmental and/or physiological processes affected by loss of the AIP protein.
- identification of small molecules that may prevent the developmental/ physiological phenotypes as potential therapeutics to improve outcomes for affected human infants.
- 5 or more publications to inform development of clinical therapeutics.

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

This project will have short term benefits for the academic research community and longer term benefits for those suffering disorders resulting from AIP mutations.

Ultimately, the predicted long term benefit is reduced morbidity for those suffering from AIP loss of function mutations and the prevention of human death.

The academic community will have short term benefits from:

Output 1. Understanding AIP's Role in Development and Physiology Using Zebrafish: How: Model Validation: Establishes zebrafish as a valid and effective model for studying the developmental and physiological impacts of AIP loss.

Increased Knowledge Base: Immediate enhancement of scientific understanding regarding how the loss of AIP affects developmental and physiological processes in a model organism closely related to human biology.

Increased research efficiency: The success of this project will illustrate the increased efficiency, and refinement in animal use, provided by the use of larval zebrafish for the study of metabolic disease.

Output 2. Identification of Small Molecules:
How:
Identification of small molecules that prevent phenotypic changes resulting from AIP loss of function will be of benefit to academics and clinicians as well as patients as it will give information regarding the mechanism of action of AIP in the body and will identify candidate molecules that can be used to develop treatments for conditions linked to AIP deficiency.

Output 3. Publication of Research Findings:
How:
Knowledge Dissemination: Immediate dissemination of new knowledge through publications, not only contributing to fundamental knowledge within the global scientific community but also highlighting the utility of zebrafish in modelling human diseases,

thereby contributing to the refinement in animal usage which is a priority to society as a whole.

Clinical Insight: Provides clinicians and researchers with the latest findings, potentially influencing current therapeutic strategies and patient care, emphasizing the relevance of animal models in translational research.

Clinicians and patients will have long term benefits from:

Output 1. Understanding AIP's Role in Development and Physiology Using Zebrafish: How: New Treatment Paradigms: Patients will benefit in the long-term from the development of novel therapies based on the molecular pathways affected by AIP, potentially leading to new treatment paradigms for related conditions.

Output 2. Small molecule screen.
How:
AIP Therapeutic Efficacy: Families carrying AIP mutations and individuals suffering from AIP- related disease will benefit from the development and eventual clinical use of small molecules identified through zebrafish studies leading to improved outcomes for infants affected by AIP-related conditions.

Preventative Strategies: Long-term application of these small molecules might also lead to preventive strategies, reducing the incidence or severity of developmental and physiological disorders.

Output 3. Publication of Research Findings:
How:
Influence on Policy and Guidelines
Clinical Guidelines: Research findings and subsequent publications can influence clinical guidelines and best practices for managing conditions related to AIP deficiencies.

Healthcare Policy: Long-term data from zebrafish studies may inform healthcare policies and funding decisions, emphasizing the importance of early intervention and targeted therapies based on model organism research.

The general public will benefit in the long term from Economic and Social Impact: How: Healthcare Cost Reduction: Effective treatments and preventive measures, initially validated in zebrafish models, can reduce the long-term healthcare costs associated with managing chronic conditions stemming from AIP deficiencies.

Quality of Life: Improved therapies can significantly enhance the quality of life for patients and their families, leading to broader societal benefits.

Overall by using zebrafish as a model organism, the project can provide significant insights and advancements in understanding and treating human diseases, demonstrating the translational potential of findings from model organisms to human health.

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

To maximise outputs, we aim to publish in well renowned journals, publicise findings via social media, university official website, national Public Engagement events and at national and international conferences. We will collaborate with other researchers in the field, including industry, to ensure realization of potential therapeutic benefits. We will work

closely with clinicians and collaborators in the field to ensure findings are transmitted directly to them.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): Adult wild type or genetically modified fish: 1000; wild-type or genetically modified larvae: 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.

It is not possible to study complex metabolic and behavioral processes such as those associated with AIP loss of function without the use of animal models.

Zebrafish are non-mammalian vertebrates that have been shown to have a translationally relevant behavioral and metabolic repertoire and are an established model to human disease phenotypes. Zebrafish have been extensively used as a genetic model for the study of development and thus a large number of resources including transgenic lines and mutants are available. Thus, they are the vertebrates with the lowest neurophysiological sensitivity likely to yield results relevant to the human condition.

Invertebrates such as Drosophila and C. elegans are other popular model systems for genetic analyses. However, despite being useful models for metabolic and neurobiological analysis, these organisms share 60 and 40 percent of the genome with humans while zebrafish share 70%.

Furthermore, invertebrate brains do not show the same level of complexity as vertebrates, and it is not clear whether the neuronal networks established to be involved in human behavioural control are present in invertebrates. Thus, findings from Drosophila and C. elegans are less likely to be relevant to human biology than findings from studies on vertebrates such as zebrafish where the conservation of neuronal networks has been established.

We use both larval and adult forms in our studies. Wherever possible, assays are conducted before 5 days post fertilisation (dpf), the age at which zebrafish are considered to be free-feeding and are protected by UK legislation. However, more complex phenotypes associated with AIP loss of function such as tachycardia and failure to thrive that may derive from, for example, deficits in later gut development, may not be evident at early larval stages due to the species specific development of the gut (some aspects of gut development do not occur until 12-14 dpf) and presence of a large supply of yolk that provides nutrients to the developing zebrafish until 6-7 days post fertilisation.

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

Animals will be bred, reared in non-standard conditions with restricted feeding in the presence and absence of test substances.
More specifically, wildtype, heterozygous and homozygous mutant animals will be bred. At 4-5 dpf wild type (AB, TU or Tupfel) or AIP loss of function mutant larvae or larvae carrying

variants in genes predicted to act up or downstream of AIP are placed individually in a well of a 48 well multi-well plate in an appropriate amount (e.g.1-2 ml) of fish water or physiological saline, or in groups of 3-5 in a well of a 24 well plate (or similar container) with an appropriate amount of water or equivalent physiological saline, in the presence or absence of a test compound for up to 14dpf with or without daily feeding for up to 3 days.

Only test compounds that are predicted on the basis of in vitro studies to improve development and survival (e.g. autophagy regulators such as rapamycin, metformin) and with no harmful effects at embryonic stages will be used.

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

AIP homozygous loss of function mutants and animals used in metabolic assays which includes temporary fasting, may show tachycardia and failure to thrive over the course of the experiment (up to 14 dpf). As the aim of these experiments is to identify potential therapeutics to prevent symptoms and improve healthy development, some animal suffering is unavoidable. However, the majority of studies will use biochemical analyses at stages before visible evidence of harm and only in the event of predicted beneficial effects on the basis of this biochemical analysis will studies be conducted at older ages.

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)?

75% of animals are expected to be used in protocols of mild severity. 17% in protocols of moderate severity, and 8% in procedures of sub-threshold severity.

What will happen to animals used in 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?

It is not possible to study metabolic processes that involves the coordination of different organs without the use of animals.

Invertebrates such as Drosophila and C.elegans are other popular model systems for genetic studies. However, despite being useful models in which to assess cell biological processes their physiology is evolutionarily distant to vertebrates and it is not clear whether the metabolic processes and neuronal networks established to be involved in human disease are present in invertebrates. Thus, findings from Drosophila and C. elegans are less likely to be relevant to human biology than findings from studies on vertebrates such as zebrafish where the conservation of metabolic processing and

physiological pathways has been established.

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

We collaborate with human geneticists and clinicians using patient derived cell and tissue cultures as well as mouse embryonic fibroblasts to examine the role of AIP and to identify potential therapeutics.

We considered experiments in mouse embryonic stem cells (MEFS), patient derived cell and tissue culture and organ on a chip approaches.

Why were they not suitable?

Although we and our collaborators have gained valuable insight into the role of AIP in metabolic processing from cells in culture, these cells do not recapitulate the full spectrum of the disease seen in human infants.

Much of our biochemical data come from mouse embryonic fibroblasts (MEFs) in culture and we use these cells to help identify potential therapeutic before progressing to animal models. However, MEFs are a single cell type and cannot give us information regarding the role of AIP in the complex environment that results from different cell types within a tissue or developing animal. We will continue to use tissue culture as the first step in our search for therapeutics.

We have also explored the possible use of organ chip technologies- QMUL has an established 'organ on a chip' research centre. However, although current organ chips can model some individual organs and in some cases multi organ systems, systemic models, as necessary to establish the in vivo and developmental function of AIP and identify potential therapeutics, are not yet developed.

Similarly, patient-derived cell and tissue cultures cannot recapitulate the full spectrum of the disorder. In addition, due to the limited number of children that survive to infancy, it is not possible to obtain sufficient cell and tissue samples to enable these studies to be conducted in patient derived samples.

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 previous studies in our lab and the lab of others in the field.

For biochemical analysis at larval (4-10 dpf) time points, studies of cell extracts suggest 5-10 mg of tissue is required per replicate. This amount of tissue can be obtained from approx 20-30 4-7dpf larvae (Kirla et al, 2018). For 3 replicates per treatment and 2 treatments per experiment (e.g. with and without drug exposure) and 2 genotypes (wildtype or homozygous) 30x3x2x2 = 360 larvae are required.

Kirla et al (2018) :_Front. Pharmacol. Sec. Predictive Toxicology Volume 9 - 2018 | https://doi.org/10.3389/fphar.2018.00414

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

We use research experimental tools like the NC3Rs Experimental Design Assistant to ensure appropriate study design and to help reduce the number of animals used. Wherever possible we conduct studies in cells in culture before experiments 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?

To minimise number of animals used we will optimise genotyping protocols to allow genotyping at early life stages to increase breeding efficiency. Pilot studies will also be undertaken for all new studies to help minimise animal usage. Wherever possible we will conduct multiple developmental and/or biochemical analyses from all animal tissue obtained.

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 zebrafish as our model species. Zebrafish are non-mammalian vertebrates that have been shown to have a translationally relevant physiology and behavioural repertoire. Thus, they are the vertebrates with the lowest neurophysiological sensitivity likely to yield results relevant to the human condition. Zebrafish embryos and larvae are transparent allowing visualisation of morphological defects at early life stages minimising harm.

The majority of metabolic studies will be conducted on tissue collected from larvae prior to free feeding stage. However, some studies aimed at identifying compounds that improve healthy development will be conducted at later stages up to 14 dpf. All compounds to be tested will be assessed in cells in culture prior to being used in larvae and only those showing promising results be taken forward to invivo testing. Compounds will be tested in embryonic zebrafish for ability to rescue metabolic effects before being tested in larvae and again, only those showing promising results be taken forward to larval stages. Treated and untreated animals will be assessed daily for evidence of therapeutic potential or harm/ distress thereby minimizing the duration of studies and suffering of the animals. All animals will be killed by schedule 1 method at the end of these experiments preventing lasting harm.

Why can’t you use animals that are less sentient?
Invertebrates such as Drosophila and C.elegans are other popular model systems for behavioural and metabolic genetic studies. However, despite being useful models for the

analysis of cell biological processes, the translational relevance of these organisms for whole organism metabolic disease phenotypes has yet to be established.

Furthermore, invertebrate organisms share less percent of the genome with humans than vertebrates such as zebrafish. Thus, findings from Drosophila and C. elegans are less likely to be relevant to human biology than findings from studies on vertebrates such as zebrafish where the conservation of metabolic processing and physiology has been established.

We use both larval and juvenile forms in our studies. Wherever possible, analyses are conducted at larval stages. However, some aspects of development, such as aspects of gut development that may be relevance here, are not evident until juvenile stages (10-14 days).

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

Refinements to minimise suffering include increased monitoring for all experimental animals, particularly following any invasive procedure (skin swabbing/ fin clipping/ anaesthesia) or drug exposure. We regularly review protocols and the use of analgesics.

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

We follow FELASA and LASA best practice guidelines (e.g. see Alestrom et al 2020) and the ARRIVE guidelines 2.0. In addition, the Principal Investigator (PI) is a member of the EU zebrafish society (https://www.ezsociety.org/), a society dedicated to promoting and improving the use of zebrafish for biomedical research. Through active participation in this society, the PI is also fully engaged with the EU wide best practice advice regarding the use of zebrafish for research (see Alestrom et al 2020).
Aleström P, D'Angelo L, Midtlyng PJ, et al. Zebrafish: Housing and husbandry recommendations. Lab Anim. 2020;54(3):213-224. doi:10.1177/0023677219869037

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

We are kept up to date with advances in the 3RS through the NC3Rs newsletter direct to personal email addresses and via Institutional notification from the NACWO, NIO, Compliance Officer, NVS and through the AWERB.

52. Biology and treatment of kinetoplastid 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

Trypanosome, Leishmania, Imaging, Drug development, Neglected Tropical Diseases

Animal types	Life stages
Mice	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?

The overall aims of this programme are, firstly to contribute to the discovery and development of improved treatments and interventions for infections caused by the kinetoplastid parasites (Leishmania and Trypanosoma) and provide refined animal models for this purpose, and secondly to examine the role of the immune system in disease as a basis for better 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?

Chagas Disease, African Trypanosomiasis and Leishmaniasis belong to the group of Neglected Tropical Diseases (NTDs), which cause significant disability and death in human populations worldwide. They are also diseases of companion and livestock animals. There is currently no vaccine available for any of these diseases in humans and available drugs are far from satisfactory in both humans and animals. All three are caused by related trypanosomatid parasites. How these parasites cause the diseases they do is still not clear. These are chronic diseases in which the immune response is involved in

damage to tissues over time and where multiple organs are affected. Understanding the interplay between the host (human or animal) and parasite in causing the disease will benefit the search for new treatments since knowledge of the immune role in disease will enable the development of therapies that cure or slow the progression of disease symptoms. The complexity of these interactions requires that animals be used in this work as in vitro (i.e. test tube-based) laboratory models cannot currently replicate the complex interactions of the body's multiple organs in the course of infection and disease. Since the parasites can hide away in many different tissues it is necessary to use animals when examining the effect of new drugs or treatments. This ensures that we can see if the drug is reaching all of the infected tissues at a level that kills the parasites.

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

The expected outputs of this project include:

New knowledge on how drugs and drug candidates work in vivo and how/when they are best used,

e.g. optimal dosing or route of administration and the time during disease progression when they will confer a clinical benefit, for all three of these diseases. We also expect to identify new curative agents for these diseases. In addition, this project will generate new understanding of the roles played by the parasite and by the body's immune response in the development of clinical disease and identification of mechanisms involved. We expect to make significant progress in elucidating the process whereby nerve cells are killed in the gut during the evolution of digestive Chagas disease, understanding this process will allow us to identify targets for therapeutic intervention. We believe we will be able to generate new knowledge about how parasites spread within the skin in complex cutaneous leishmaniasis. These advances will allow new avenues of research to be undertaken in the immunology and pathology of diseases caused by the kinetoplastid group of parasites.

There will be multiple peer-reviewed publications as an output of this work and the identification of drug candidates that cure animals and can be taken forward towards clinical trials. Drug candidates that do not work will be removed from the pipeline.

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

In the short-term increased knowledge of the biology of these diseases gained during this project will be of benefit to scientists working on these diseases, and the wider scientific community, in particular to those studying the role of the immune response in chronic disease processes and will inform the discovery of new treatments. Mechanisms identified here may also be relevant to other chronic diseases, for example understanding the role of inflammation in cardiac Chagas disease may have relevance to other forms of chronic heart failure.

In the longer term, the development of new therapies for Chagas disease, leishmaniasis and African trypanosomiasis has the potential to benefit millions of people in low- and middle-income countries.

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

All basic research findings will be communicated through open access peer-reviewed publication and conference presentations. Protocols, parasite lines, models, data and refinements will be made available to the wider research community, datasets will be

deposited in public databases where these are available. We will also provide archived tissue samples to other research groups for their work.

Successful drug candidates will be advanced to further pre-clinical studies by our collaborators. Negative results or unsuccessful approaches will be incorporated into publications and will be presented at scientific conferences to ensure that these approaches are not repeated by other groups.

Species and numbers of animals expected to be used

- Mice: 5900

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 characterised model for the kinetoplastid diseases (Chagas disease, African trypanosomiasis, leishmaniasis). We and others have previously shown that they develop pathology similar to the human disease. All three of these diseases rely on a mature mammalian adaptive immune system for clinical disease to happen, and for control of parasite burden. This is not present in embryos or neonates. It is also absent from insects and fish. Consequently, for these models to be of value as models of human disease, adult mice are required. Since the parasites can hide away in many different tissues it is necessary to use animals when examining the effect of new drugs or treatments. This ensures that we can see if the drug is reaching all of the infected tissues at a level that kills the parasites and whether the drug can eliminate all of the parasites.

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

A mouse will typically be infected with a kinetoplastid parasite. The course of the infection will be followed by bioluminescence imaging for the trypanosomes. To do this, we inject a chemical agent into the infected animals that reacts with a protein in the parasite to produce light. This procedure allows us to follow parasites non-invasively in living animals using a specialised camera. The administration of the chemical will be repeated several times during the course of the infection. In the case of cutaneous leishmaniasis the development of the infection will be monitored by measuring the growth of the lesion using a caliper. During the infection course the animal may be treated with a drug or an immunomodulatory substance via one of several routes (e.g. oral, intravenous, intraperitoneal). The animal will be killed at the end of the experiment and blood and tissue samples will be collected for study and stored if not used immediately.

Experiments with T. brucei will typically last 1-2 months while those with T. cruzi and Leishmania are usually longer, typically several months.

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

In most cases we expect that the animals will experience no more than mild, transient discomfort during the procedures. Infection with T. cruzi is largely asymptomatic in mice as the pathology takes months to develop into a clinical form. In some cases, we may observe complications which could include irritation at the site of injection, and stress

symptoms from infections. Animals may show minor symptoms such as mild bristling of their coat in the acute phase of the infection when parasite numbers are at their peak but these will resolve rapidly when the immune system removes most of the parasites. After this point animals will usually show no signs of discomfort. In most experiments, animals will be treated prior to development of symptoms. In the case of cutaneous leishmaniasis we have to allow the skin lesion to develop prior to treatment because the broken skin may affect the efficacy of the drug being evaluated. Cutaneous lesions in leishmaniasis are usually painless.

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 most mice (~85%) to have mild severity with approximately 15% experiencing moderate symptoms.

What will happen to animals used in 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?

Outcomes of drug research differ between in vitro and in vivo situations as in vitro systems lack the complexity of pharmacokinetic and immunological parameters, organ systems and tissue compartments that are only achievable using in vivo models. It is known, for example, that an active immune response is required for antileishmanial chemotherapy to cure visceral leishmaniasis. Animals are also necessary for studies of vaccine and immunotherapies since an intact immune system is required to generate a response to the vaccine/therapy. The response requires the participation of multiple cell types in a range of organs. Experiments aimed at understanding how disease occurs require whole animal in vivo studies because the host immune response plays a major role in tissue damage. It has been demonstrated that the mouse model recapitulates features of human Chagas disease including both cardiac and digestive forms which cannot be replicated in vitro at the moment. Similarly, mice develop both stages of African trypanosomiasis and both cutaneous and visceral forms of leishmaniasis.

Parasites used under this license can lose infectivity or virulence during extended in vitro culture and some of them cannot easily be cultured in vitro in the most appropriate life cycle stage. To date there is no system established to fully replace animals for passage (sustaining) of parasites to maintain infectivity. Although for small scale in vitro experiments Leishmania parasites can be passaged through macrophages this is not a viable option to provide standardized, infective inocula for large scale and in vivo experiments. Therefore, animals are required to maintain parasites in an infective state and ensure experimental reproducibility.

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

Whenever possible and scientifically justifiable, questions are addressed in vitro using cell lines (for example, studies of parasite internalisation and escape from host cells), and the development of innovative in vitro systems and tissue models is ongoing. Genetically modified parasites are first characterised in vitro to prioritise which ones go forward to in vivo work.

We have investigated the use of stem cell derived organoids and in vitro cultured tissue models for some analyses where useful data may be obtained from such studies.

We search the NC3Rs database of alternatives (https://www.nc3rs.org.uk/3rs-resources). We also look at other 3Rs websites regularly for information on replacement technologies such as the USDA site (https://www.nal.usda.gov/animal-health-and-welfare/animal-use-alternatives) and EURL ECVAM (https://joint-research-centre.ec.europa.eu/eu-reference-laboratory-alternatives-animal-testing-eurl- ecvam_en). We consulted the EURL-ECVAM status report 2023 on Non-Animal Methods in Science and Regulation (https://publications.jrc.ec.europa.eu/repository/handle/JRC136460). We search

PubMed daily for any papers on trypanosomes and Leishmania which would include novel model systems and technologies. We also search preprint servers (e.g. BioRxiv and MedRxiv) to ensure we are aware of likely advances as early as possible.

Why were they not suitable?

Outcomes of drug research differ between in vitro and in vivo situations as in vitro systems lack the complexity of organ systems and tissue compartments that are only achievable using in vivo models (for example drug metabolism occurs mainly in the liver), and it is known, for example, that an active immune response is required for antileishmanial chemotherapy to cure the disease. Animals are also necessary for studies of vaccine and immunotherapies since a mature immune system is required to generate a response to the vaccine/therapy. The immune response requires the participation of multiple different cell types in a variety of organs (for example lymphoid tissue, bone marrow, the spleen and thymus). Experiments to find the mechanisms of disease require whole animal in vivo studies because the host immune response plays a major role in tissue pathology and pathology varies between different organs. Furthermore, the processes for each of these diseases are chronic, progressive and tissue specific and as a result are not currently amenable to in vitro analysis.

Current stem-cell derived organoid technology does not allow the integration of both nervous and immune systems into tissue organoids and so is not at the stage where it can replace animals yet. However, we are monitoring progress in this field closely and we have a collaboration to develop human induced pluripotent stem cells (iPSC) gut organoids incorporating the enteric nervous system and immune cells to use for Chagas disease research.

The replication rate of these kinetoplastid parasites changes during chronic infections and metabolically distinct forms can exist in different tissues. These events cannot be replicated in in vitro culture at the current time. Current in vitro tissue technologies such as organoids have a limited life span which mitigates against their use in the study of chronic and progressive diseases.

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 our previous two licences. We have data from previous work showing that the data obtained from mouse experiments does not vary much between in-bred mice, as the mice are very similar genetically. We can therefore use quite small numbers of individual mice within each experimental group and we use the same control group for multiple experiments whenever possible. The majority of the mice (4,000) will be part of objective 1 which involves the pre-clinical efficacy testing of novel anti-kinetoplastid therapies. This represents an upper estimate as the total required will depend on the number of novel treatments and regimens developed within the five-year period of the licence.

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

We employ NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental design, practical steps and statistical analysis using the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods. We will use the EDA diagram and report outputs to support experimental planning with animal users.

Additionally, PREPARE and ARRIVE guidelines will be used to assist in the planning of experiments and in the reporting of animal experiments, respectively.

Genetically altered parasite strains will be prioritised using in vitro analyses to reduce the number of studies requiring in vivo experiments. Whenever possible GA strains will be genetically tagged (barcoded) so multiple parasite strains can be used in a single animal thereby reducing the overall number of animals.

All experiments are planned in order to maximise experimental output with the least number of animals used and to minimise bias by allocating animals to treatment groups in random order. Whenever possible data is evaluated by a researcher either not involved in the animal work or unaware of the nature of treatment given to each group.

Control groups of mice (e.g. untreated in drug activity studies; uninfected animals in pathology studies) will be included and whenever possible animal numbers will be limited by sharing control groups or using control groups for analysing multiple outcomes.

Additional data will be generated on e.g. transcriptomics, tissue pathology and immune parameters in selected experiments to enrich the scientific output of this programme of work. Whenever possible this data will be generated using animals undergoing drug treatment or control groups to limit animal numbers. Tissue samples are also archived for future use. These samples can prevent the need to carry out new experiments and therefore reduce the number of mice needed for the research programme overall. We have collaborations with several institutions for sharing of tissue and blood samples, RNA, DNA so that the maximum data can be obtained from experiments.

The application of non-invasive bioluminescent/fluorescent imaging represents a major step forward in terms of reduction and refinement and following the successful establishment of imaging techniques for T. brucei and T. cruzi infections in mice we are setting up in vivo imaging techniques for specific species of Leishmania parasites. In vivo imaging allows the longitudinal assessment of parasite infection in an animal and allows us to quantify the number of parasites in individual animals accurately over time without killing the animals, consequently leading to a reduction in the number of animals used. We have developed a dual reporter system with bioluminescence and fluorescence so that maximum data can be obtained from each experimental animal, thus reducing the number required further.

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 use in vivo bioluminescence imaging with trypanosome infections. This allows the parasite burden in individual animals to be measured accurately over time without killing the animals and significantly reduces the number of mice required for each study.

Whenever possible, experiments will be run in parallel (for example when assessing the effects of novel drugs) so that two experiments can utilise the same control groups thus avoiding unnecessary duplication and use of extra mice, and GM strains of parasites will be barcoded so multiple parasite strains can be used in a single mouse, thereby reducing the overall number of animals.

Tissue samples are collected and archived for future use in experiments that may become necessary. These samples can prevent the need to carry out new studies and therefore reduce the number of mice needed for the research programme overall. We have collaborations with several institutions for sharing of tissue and blood samples, RNA, DNA so that the maximum data can be obtained from experiments.

Where new strains of parasites are to be used a pilot experiment of no more than three animals will be run to assess the virulence and dynamics of the infection so that highly virulent and potentially lethal strains can be excluded.

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 mice which are widely used models of kinetoplastid infections, and it has been demonstrated previously that they develop similar clinical pathology to humans and are therefore appropriate for these studies. We have a wealth of long-term experience of these infections in animals and the course of infection is predictable allowing us to increase monitoring during phases when we expect some symptoms to show, such as the peak acute phase when parasite levels are at their highest. This predictability also minimises the possibility of unexpected pathology ensuring that experiments derive

maximum outputs from the minimal number of animals used, and that no unanticipated adverse effect arises, hence avoiding unnecessary pain and suffering for the animals.

We use bioluminescence monitoring of parasite numbers in animals over time so we can quickly identify if the parasites have increased replication or virulence and then intervene before pain or suffering occurs. Most of our work is to study the chronic infection stage and so the animal has to pass through an unavoidable acute phase to reach the chronic phase.

Chronic infection with T. cruzi is often asymptomatic in mice, even while clinically relevant pathology is developing, mice infected with T. brucei will develop minor and transient symptoms which mirror the undulating nature of the human infection. Visceral leishmaniasis is often asymptomatic in mice during the period in which experiments will run. In the case of cutaneous leishmaniasis the lesion has very different properties to the skin around it and so the lesion has to be allowed to develop prior to drug efficacy testing or analysis of pathology, however cutaneous lesions in leishmaniasis are usually painless

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

The experiments require a host that is similar to humans and has an intact and mature mammalian immune response. Both the control of parasite burden and the genesis of pathology requires the immune response. Alternative model organisms (for example, zebrafish) are not infected by these organisms and do not have the appropriate immune system. In addition, these infections are chronic and multi-organ so require adult animals.

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

We use non-invasive bioluminescent imaging where possible to monitor the parasite burden. This allows us to see very rapidly if a novel treatment is not clearing the parasites and so the experiment can be terminated before the animal develops clinical symptoms. We can also identify parasite lines that are not responding to immune control using this method and so terminate experiments early and then remove these strains from further experimentation. For Chagas disease we also use ECGenie non-invasive cardiac monitoring for some experiments which allows us to identify animals that may be developing cardiac problems.

For drug efficacy experiments we also routinely use parasite strain/mouse combinations that are less pathogenic (e.g. CL-Brener strain of T. cruzi in BALB/c mice) so that we can assess drug efficacy in a model with minimised welfare costs.

There is regular close monitoring of all animals by scientific and animal care staff on a daily basis. Animals will be handled using non-aversive methods (tunnel and/or cupping) to reduce distress during procedures. Where interventions such as a new substance administration might increase the risk of adverse effects, monitoring of animals will be more frequent to assess for any unexpected pain, suffering, distress or lasting harm.

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

We will use the ARRIVE 2.0 and PREPARE guidelines and will also attend NC3Rs training courses or workshops where appropriate. We will regularly monitor 3Rs websites such as the NC3Rs, USDA and Norecopa for advances in refinement techniques. We will refer to the most current LASA guidelines (http://www.lasa.co.uk/publications/). We routinely carry out literature searches for work on the particular organisms we are interested in so we can

be rapidly apprised of refinements to animal models and developments in alternatives such as in organoid or 3-dimensional tissue culture methodologies. We will also monitor the 3Hs website for advancements in animal handling and housing techniques that reduce distress and aversion in the animals. Administration of substances will be guided by the document "Refining procedures for the administration of substances: Report of the BVAAWF, FRAME, RSPCA, UFAW Joint Working Group on refinement

(https://journals.sagepub.com/doi/pdf/10.1258/0023677011911345)" to ensure minimal distress to the animals.

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

We will regularly check information on the NC3Rs website and we have signed up to the NC3Rs newsletter. We also look at other 3Rs websites such as the USDA site (https://www.nal.usda.gov/animal-health-and-welfare) and Norecopa (https://norecopa.no/). We will seek to implement any recommended advances. We will routinely carry out literature searches and searches for new 3Rs related websites to ensure that we are up to date. Our BSF staff also routinely inform us of NC3Rs guidance and advances in the 3Rs.

53. Patient Led Identification of Novel Antibody 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

Key words

B cells, Cancer, Antibodies

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 what antibodies patients have produced which led to an exceptional complete or prolonged positive response to their disease, which may be developed into therapies for the much greater proportion of patients who have not experienced such a 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 identification of novel, disease relevant antibody-target pairs may yield therapeutic insights into unknown disease biology permitting direct biotherapeutic development of a protective antibody, or new drug discovery against a novel disease target. This is an innovative approach which is focused on non- biased drug discovery, guided by the individuals' immune responses rather than a classical drug discovery approach (the latter which identifies therapies based on a prior knowledge of the target).

This allows research into previously categorised "hard-to-treat" diseases such as pancreatic and brain cancers. We anticipate this approach has the potential to build our

understanding of the fundamental biology of cancer, as well as having the potential to deliver much needed therapeutic options.

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

Outputs will include but not limited to

- Increased understanding of how autoantibodies may affect disease progression in cancer

- Findings and results shared exchanged with the clinical centres, physicians and academic leaders where the original patient donor samples were first obtained

- Published findings in peer reviewed journals, at scientific conferences and seminars

- Introduction of novel antibody based therapeutics in human cancers for improvement of human health

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

First, and foremost, the patients themselves. Due to the advantages of identifying potential antibody therapeutics first in patients, this approach considerably speeds up potential introduction of novel therapies into the clinic. A rough estimate would be 3 years, as opposed to a more traditional 5 years as is currently characteristic from "bench to bedside", and so could occur within the span of the proposed PPL.

During this time, results generated from these models greatly assist confirmation of artificial intelligence assisted antibody prediction modelling. Understanding, and more importantly, predicting how antibody chain pairing occurs during recombination events is a subject of intense research in medicine and scientific research, on a similar level of Artificial Intelligence being used to predict protein folding structures. We hope to continue to publish and share results as they occur from these studies within this timeframe of the Project License.

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

We maximise efforts to produce key therapeutic antibodies by confirming their presence not only within the originating disease indications, but across disease indications. We do maintain a continually growing database of B cell receptor antibody signatures, and are able to nominate potential therapeutics based on more than one patient and/or indication. When candidate antibodies are confirmed in more than one patient donor, this considerably builds confidence that the antibody candidate could have a significant positive impact in a clinical setting.

Collaborations (both academic and industrial) are an integral part of all research programmes into novel therapeutics. We will continue to share strategies, results (both positive and negative), and next steps with our collaborative partners and through active publishing in peer reviewed journals.

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.

Adult mice are used in cancer studies because they are more like humans when cancers occur. These mice can get tumours on their own, similar to how cancer develops in people. We also may study special mice that are bred to have specific cancer-related traits. This will allow us to understand how cancer develops in animals, which will directly relate to the majority of cancers in humans.

The work we will do is not repeating others work, but adding new knowledge, and the results are more likely to be useful for finding new effective medicines for humans.

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

We have two main areas (2 procedures) in our research which require the use of mice in finding new potential medicine in oncology

● Pharmacokinetics: Understanding how long a new antibody medicine will stay at an effective concentration effective in the body after injection (1 procedure)

● Cancer Therapy: Treating cancers in mice with new therapeutic antibodies (1 procedure) Pharmacokinetics:

As we are researching new medicines, we need to be sure that the potential antibody medicines will stay in the body long enough to be effective. One of the ways to test this is to give a similar (based on weight) dose amount into mice. This could be via an injection under the skin, or directly into the circulation (This procedure is usually no more painful than an injection one might receive at a doctors surgery). Over a period of days or weeks, small blood samples are taken from these mice, to examine if the antibody therapy is still present. This study directly helps us to understand how helpful such a medicine would be for human patients.

Cancer Therapy:

Finally, the goal of the research is to find an effective antibody medicine which will inhibit or stop the growth of a cancer. We do this by a small injection under the skin of the flank, to either place cancer cells or small pieces of cancer tissue and allow to grow. When the cancer begins to grow, we will give groups of mice (calculated by statistics so that the results are both meaningful, scientifically relevant, and will limit the future requirements for repeat experiments) therapies:

1. Medicines known to affect tumour growth (positive controls), and compared to

2. New candidate antibodies.

Antibodies are given by injection under the skin, into the circulation, or directly into the tumour itself. These injections are similar to a needle injection one may experience at a hospital or doctors surgery. Typically, antibody treatment occurs 1-3 times a week, with

volumes not exceeding 15% of the total body weight of the mouse (for example, for a 25g mouse, a typical injection volume would not exceed 250ul) Tumour growth, respective to each therapy, is then carefully monitored and measured, so that an understanding of how effective the medicine might be to patients is gathered.

The growth of the tumour is typically painless, although the mice will be monitored daily for any signs of distress, or if there is any signs that the tumour have been wounded by daily activities, or if the growing tumour breaks through the skin (ulceration). In the case where the growing tumour has been exposed through the skin, mice would be removed from the trial and humanely killed.

The cancer growth trials are ended either when the tumours reach a specific size (no more than 10% of the mouse's body weight as per the Workman guidelines), or according to a pre specified length of time before this growth limit, which is also guided by pre-established growth rates respective of the cancer lines being assessed (which may differ from 3-6 weeks). At this point, all the animals are humanely sacrificed, and tissues and organs are removed for comprehensive study for the effects of their respective treatments.

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

During Pharmacokinetics: mice are expected to experience two effects. The first, an initial injection (beneath the skin, or directly into the circulation) of the antibodies to be tested. Discomfort is transient and not expected to cause any distress above that experienced with a needle injection. Blood sampling will occur at points during the time periods following the initial injection. Blood will be in small volumes and will not exceed 10% of total blood volume overall during the course of the protocol. Blood sampling, following established protocols and guidelines, is not expected to cause any lasting harm or distress.

For Cancer Therapy: cancer cells may be injected subcutaneously by needle injection and not expected to cause any more than a brief discomfort as is associated with a needle injection. Small sections of tissue may be implanted under the skin, this will be performed under general anaesthesia following a small incision (less than 1cm) to implant the tissue. The incision will be closed and pain managed with analgesic, which is expected to be brief and transient, lasting no more than a day or two. Mice will be monitored for any signs of distress during this time. Mice undergoing this protocol may receive one or the other (subcutaneous injection of cells or implantation of a small tissue section), but not both.

The growth of the tumours overs days to weeks following implantation is not expected to have any lasting harms, though may provide minor discomfort. During ensuing growth, additional health checks are routinely undertaken to ensure that any additional health concerns are immediately addressed.

This may include weight loss, decrease in appetite, accidental wounding to the growing tumours, and potential ulceration. These events while rare will be addressed immediately, either by treatment with analgesic, or soft food diet supplements.

Risk of tumour ulceration generally occurs as a result of one or more of the following: 1) The accelerated growth rate of cancer cell lines may rupture through the skin; 2) Known cancer models which have been reported in literature to be prone to ulceration (for example, the kidney cancer cell line RENCA); and 3) Anti-cancer medicines which either directly or indirectly initiate tumour reduction. This growth reduction is typically the result of dying cancer cells, and a direct indication of a potentially effective medicine. During this

treatment, skin ulcerations may appear on or near the tumour site, and in any case where the wound is raw or inflamed, mice will be removed from the protocol immediately and humanely killed to ensure that no prolonged pain or distress 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)?

Mild Severity: 40%

Moderate Severity: 50%

What will happen to animals used in 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 for mouse models could be summarised in 5 points.

1. Recreating the complex tumour microenvironment

2. Modelling multiple aspects of immune system interactions, which involves multiple organ systems

3. Potential effects (positive or negative) of metastasis, which seeds more than one systemic location

4. Physiological relevance of a whole-body system in cancer - the heterogeneity of cell types (cancer and non-cancer) involved

5. Drug metabolism or toxicity

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

Our approach take patient produced antibodies and predicts antibody sequence and specificity based on artificial intelligence analysis before we even produce the antibody, to increase the chances of success. Following prediction modelling, we use extensive cell culture (migration, invasion, proliferation), cancer cell spheroid assays, organoid cultures, and complex human protein arrays.

It is important to note that each of the above are extremely valuable (and performed) in the work that we do to study the cellular interactions and effects of these new potential antibody therapies.

Why were they not suitable?

While in vitro cell cultures and organoid systems are extremely valuable for preliminary screenings and understanding basic cellular processes, they lack the systemic complexity and physiological relevance provided by in vivo mouse tumour 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.

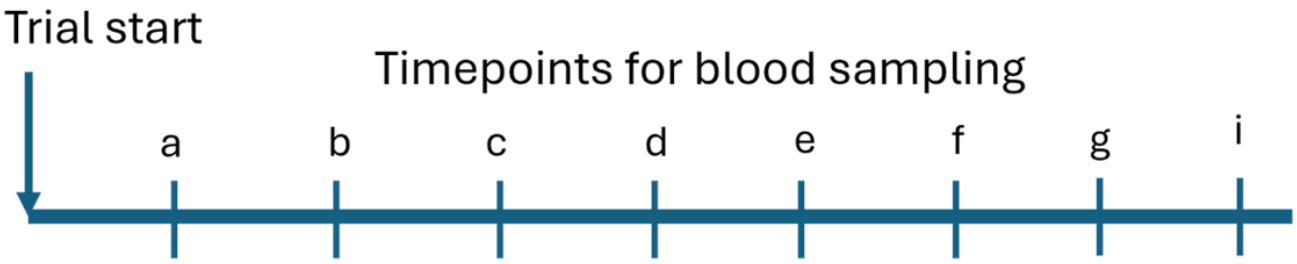
How have you estimated the numbers of animals you will use?

Pharmacokinetics: Usually 12 mice per group, over 7 days, per test of novel therapeutic antibody

(Testing exposure of therapeutic antibodies): Over the course of the experiment, blood sampling will occur to measure levels of injected antibody in circulation. This is controlled by standard non-human reactive control antibody, which has a known concentration over time, and is compared to the test therapeutic antibody to provide an understanding of how long it remains in circulation. Per sample time point during the course of the protocol, 3 animals are required to be bled. Variations in the concentration of injected antibody are expected to be consistent with each other, which allows for the small groups of three. In keeping with minimising animal harm, the maximum number of times a single mouse will have blood samples taken, in this protocol, is three.

So that all data points can be collected, groups of three will be staggered at points along the experiment which will allow for multiple sampling points yet ensuring that no single mouse will have blood samples taken more than 3 times.

A typical experiment looking at antibody exposure in mice runs over the course of 7 days with 8 sampling points. Grouping mice first in treatment groups, and then subdividing them into sample groups within their cohort, typically requires 12 mice per antibody to be tested. In most cases there will be one test antibody cohort, and one control antibody group.



Cohort X = 12 mice	Sample times
Group 1 (3 mice)	a, d, g
Group 2 (3 mice)	b, e, h
Group 3 (3 mice)	c, f, i

Tumour implantation and treatment with therapeutic antibodies. (10-12 mice per treatment group)

When investigating potential therapeutic antibodies which may control tumour growth, the typical growth suppression which would signal that the test antibody may yield success in the human clinic is measured against a 1) negative control group, which is expected to exhibit no change in tumour growth, and if available, 2) a positive control drug with a known effect. This positive control effect usually reflects about a 50% response rate in specific mouse models of cancer, which aid in the interpretation of the test antibodies. The test antibody or antibodies, would be expected to suppress tumour growth greater than 20-100% of the negative control group.

From well-established studies of cancer in mice, the predicted effect sizes are quite large (from 2-6 times the reported standard deviations of most mouse cancer studies). In addition, using the inbred strains of mice, receiving the same cell line, and the exact same treatments, makes predicted sample sizes for looking at the effects appear quite small (usually from 4-6 mice per treatment group).

However, there is a real danger of proceeding with these small sample sizes despite the predicted large effects for three reasons.

The first is to address spontaneous rejection of injected or implanted tumour tissue. While infrequent, this does occur in about 15% of mice injected (though varies according to mouse strain and cancer lines).

The second reason concerns having good experimental groups all at a similar stage of tumour development when treatments occur. Ideally, all tumour bearing mice will have equivalent tumour volumes at the start of treatment (typically, about~60mm3). Having equivalent starting volumes across the mice is required prior to randomisation of mice, and allocation to treatment cohorts. Roughly 30% of the growing tumours grow faster or slower than the expected rate, and this is influenced again by mouse strain (strains and models are not mixed during trials) and cancer cell line.

Finally, there are potential adverse effects, or unexpected events in addition to the above.. This includes unexpected loss in numbers (such as tumour ulceration, wounding, unexpected mouse sickness).

Considering all these variables mentioned, enough numbers need to be included so that the risk of having to repeat the protocol is minimal.

Example:

Assume 4 cohorts of mice are needed to assess impact of a novel therapeutic in tumour bearing mice. We would expect to observe a >25% growth difference between control groups and treatment group: 4 Cohorts x 3 mice per cohort = 12 mice

However If 12 mice are subcutaneously injected with cancer cell line 12-15% (Tumour line fails to grow) = 10 or 11 mice

Before treatment can be started, all tumour volumes need to be roughly equivalent.

10 mice - 30% variable growth rate which is not suitable for randomisation prior to allocation to treatment groups = ~7 mice in total for 4 treatment groups.

From this example, the trial would have to be abandoned due to lack of the numbers required for a meaningful result.

In conclusion, we would require at least 10-12 mice per treatment group, so that we have at least 5-6 mice available during, and at the conclusion of the trial, thus greatly minimising the risk of repeating failed experiments due to insufficient numbers.

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

Consultations with the NC3R's experimental design guidance, current guidance from published and controlled studies with similar aims (for example, Workman et al 2010 "Guidelines for the welfare and use of animals in cancer research"), proper randomisation of groups, blinding, and have utilised statistical methods for ensuring that the numbers required are minimum and have the least likely chance of having to perform any repeat experiments.

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

For experiments where a cancer cell line or tissue is used, to ensure that the cells or tissues are pathogen free (cell lines are checked by regular internal, lab based screens, and external genetics screens) and cultured by in the appropriate sterile conditions. This minimises the risk of bacterial or fungal infection of the cell lines to be injected, and significantly reduces the chance of generating false results.

In addition to the main goals (antibody concentration and tumour growth rate suppression), tissues and biofluids are usually taken at the end of each experiment and thoroughly examined for additional insight into how these potential therapeutic antibodies may work. If we understand what the medicines are doing, we can more accurately reduce the reliance on live animal models and better recreate those conditions using lab-based 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.

Model: Mice

The methods proposed are ones which are routinely used in cancer research (such as injections with a needle, implantation of subcutaneous cell suspensions , and blood sampling). However, all techniques continue to be re-evaluated and investigated for improved methods which reduce animals harms. The majority of the methods involve a needle injection similar to which is experienced in the doctors surgery.

Any animal distress caused by tumour growth and treatment will be carefully managed by daily health and welfare checks.

In certain cases, it may be necessary to surgically insert a very small section of tumour tissue into the flank.

In order to reduce animal stress, implantation of small piece of tumour tissue using a small (<1cm) incision beneath the skin is carried out under general anaesthesia, and pre-emptive analgesia is used to control pain, and afterwards during follow up welfare checks.

The size of the implanted tumour tissue is usually equal or less than 0.5cm. The reason for the size, is to have a tumour which more accurately looks like, and a similar size found in human patients when diagnosed.

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

Cancer is heterogenous. It represents the interaction of several organ and tissue systems, which can be challenging to model in vitro. Mice, and in particular, inbred and genetically modified strains, represent a way that complex interactions of cancers can be tested with new potential medicines.

For determining how long a potential therapeutic antibody can remain in circulation, this also requires complex systems which can address ADME (absorption, distribution, metabolism, and excretion), which in turn, will aid to calculating relevant dose in patients.

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

For pharmacokinetics, animals will be checked daily for any signs of distress or ill health. Mice have been limited with respect to the number of blood samples that can be taken, and the time between sampling purposely spaced and limited so as to minimise potential distress. We will also continue to investigate accurate microsampling techniques which further reduce the volume of blood taken without the loss of data.

For the implanted tumour treatment trials, this will continue to be a process that we will work to maintain high standards of welfare and minimise distress. To assist with recognising signs of distress or pain, we have included a list of recognised end points which will be used to assist with overall recognition of general condition and well-being on this trial.

Pain management will be addressed with analgesics. Supportive treatment such as soft food mixture or food gels will also be used when appropriate as well as maintaining environmental enrichment, and minimising cage and animal handling.

Technical expertise for delivering the protocols will be routinely reviewed and training records maintained. Maintaining staff who are both comfortable and skilled with the techniques involved reduces potential associated harms.

On a more general level, we will also review, and take into account the expertise and advice from the establishment staff on improvement to environmental enrichment, and diet.

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

We will review our protocols using both PREPARE and ARRIVE guidelines and stay abreast of current research which may also assist in the 3Rs with respect to our proposed numbers.

We also refer to Workman et al, 2010 on determining guidelines for the research proposed with respect to sample volumes, growth rates etc.

We will also continue to develop a good working relationship with our designated primary site NACWO, NIO, NTCO, NVS and animal technicians.

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

We will continue to stay informed of news, guidelines and advice on the NC3Rs site. This includes regularly consulting the NC3R Resource Library, and Experimental Design Assistant

We will also continue to develop a good working relationship with our designated primary site Named Animal Care and Welfare Officer (NACWO), Named Information Officer (NIO), Named Training and Competency Officer (NTCO), Named Veterinary Surgeon (NVS), and animal technicians.

54. Understanding how the manipulation of barrier's properties influences the transport of molecules across the biological membranes

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

Biological barrier, Skin, Nail, Biomarkers, Chemical absorption

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

This project aims to get a better understanding of how the integrity of externally facing biological membranes such as the skin, the buccal membrane (i.e. the inside of the mouth) and the nail, impact the passage of molecules into the body and how manipulation of some the characteristics of these membranes affects the transport and activity of the molecules.

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 important to understand the how substances cross externally facing biological barriers (such as the skin) as this knowledge underpins how we interpret chemical exposure. This chemical exposure can be unintended, i.e., environmental, or intended, i.e., for a medical treatment. Scientists use knowledge of the absorption of chemicals into and through

externally facing biological barriers to predict biological exposure. This helps in the safety assessment of chemicals, and also in the development of new chemicals that we want to administer into the body for a medical need.

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

Through the project's objective of 'understanding the transport of chemicals across the skin with and without the use of a medical device', the studies will uncover the fundamental anatomical features and tissue physiology that control the tissue's permeability. This will facilitate the development of novel approaches to enhance the delivery of therapeutic agents in a needle-free manner. Similarly, the project's objective of 'Understanding, using skin stretching, the extraction of chemical out of the skin' allows the development of non-invasive methods to characterise changes in the tissue in both health and disease state.

The project will also look into how the therapeutic agents penetration occurs in the presence and absence of the wounds with the aim to further understand wound healing and potentially facilitate the development of novel treatment for wound healing. In addition, the project will investigate therapeutic agents penetartetion across nail tissues for the treatment of nail infection. The output from this work will include advancement of the current understanding of nail permeation and the development of novel device to optimize treatment efficiency.

These findings will be disseminated in scientific conferences, published in scientific journals and provide the scientific community with key information that is needed for development strategies for early diagnosis of diseases and to develop new medicines (short-term within the time frame of the project).

It is anticipated that upon the completion of this project, several new approaches and patented devices will be developed to both administer chemicals into externally facing biological barriers, e.g., the skin, the inside of the mouth etc, and extract chemicals (biomarkers) out of these tissues.

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

Short to medium term benefits: Several new non-invasive or minimally invasive approaches will be developed during this project for both the administration of therapeutic agents to topical biological barriers (such as the skin and nail) as well as for the extraction of biomarkers across topical barriers such as the skin. For example, a device that applies suction onto a small area of the skin and stretches the tissue will be developed and this will allow the movement of chemicals out of the tissue. Clinicians and scientists could use these approaches in the development of new medicines and to better understand chemical exposure both for clinical studies and in vivo animal studies (device patent filed, Apr 2024).

Long-term benefits: Once established, the approaches developed in this project, which are minimally invasive, could be used in both the clinic for both direct non-invasive biomarker sampling from biological membranes and for needless-delivery of therapeutic agents. Thes approaches could also be used for animal scientific research as a needle-free alternative for both as single or repetitive sampling thus refining and reducing animal use (long-term).

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

The outputs of the work will be maximized by combining it with both in vitro experiments to better understand the mechanistic elements of the project and clinical work to exemplify the translation of the knowledge into humans. This will generate high impact scientific

publications that have the most chance of disseminating the projects results and translating the findings into further work.

Species and numbers of animals expected to be used

- Mice: 1500
- Rats: 1600
- Guinea pigs: 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.

We will use adult mice, rats and the Guinea Pig models to study chemical transport across biological barriers. All these models have been used previously with hundreds of publications documenting the topical application of chemicals to different externally facing barriers in these species. In addition, in these animals it is possible to understand the total body exposure of chemicals in a manner that translates to humans. The different animals have been selected based on their ability to replicate the barriers in humans and or knowledge of the key differences between the biological barriers in the animals and humans. The availability of literature to substantiate the models has also been important to select the animal models in this work as models that are well established and used by scientists are more efficient to use and data generated from them is easier to interpret.

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

Chemical barrier penetration - In this study, therapeutic agents will be administered through topical application e.g., to the skin, using novel devices, e.g. a skin stretching device, a microneedle device etc. The level of the therapeutic agents deposited in the skin and that crossed the skin into the blood will be determined and the novel devices will be compared against more traditional routes of administration such as injection (e.g. intravenous, subcutaneous and intradermal) or oral administration. The devices tested will be minimally invasive or non-invasive and hence they will be administered under brief general anaesthesia (typically 30 min). The animals will be monitored closely to ensure normal behaviour is regained after the anaesthesia sessions. Non-invasive imaging may be used to track the delivery and distribution of the administered therapeutics agents. In the case where Vitamin D (as a therapeutic agent) is delivered across the skin using the novel device, its effect will be compared against the naturally synthesised vitamin D in response to UV light.

Extraction of chemicals across biological barriers- Chemicals that can help understand normal physiology and disease pathology will be extracted out of the body using novel approaches. For example, stretching the skin using a small device, by applying mild suction for up to 20 min can extract both small and large chemicals. This approach has been used previously in animals as well as in human participants in clinical studies and is known to cause no damage to the skin.

Local inflammation models- To understand how the properties of biological barriers change during disease two types of inflammation models will be used in this project. The first model consists of inducing minor skin surface damage by applying consecutive tape

strips. This is a well-known technique widely used in skin research in animals and humans. It does not cause any pain nor any visible changes to the skin. However, it will cause changes in the barrier permeability and the biomarkers released in the skin and the blood. The second model is of local inflammation (i.e. swelling and redness) to either the paw (by injection to the footpad) or the ear (by topical application to the ear's skin) but never both. The inflammation symptoms are expected to occur rapidly and recede after 6-8 h. Using novel approaches, the barrier properties and changes in the tissues will be monitored.

Superficial Tumour models- Superficial tumour animal models (e.g. skin cancer) will also be used to understand chemical penetration across the tissues and disease pathology. These models are well- characterised and will allow us to understand the changes that occur in the tumour local environment and the overlying skin tissue at the different stages of the progression. The tumours in these models are superficial and can be monitored for growth in a non-invasive manner by measuring their size. Non-invasive imaging may be used to track the delivery and distribution of the administered therapeutics agents.

Nail infection models - The project will investigate how infection of the biological barrier alters chemical penetration using an example of the nail infection. A Guinea Pig model of nail infection is similar to that occurring in humans, will be used. Both large and small chemicals, some of which will be gases and thus applied using a medicated artificial nail, will be investigated. The impact of the infection on the biological barrier and the chemical penetration will be investigated.

Wound healing models- Wounding damages the properties of externally facing biological barriers and so wound healing models will established investigated. These models are well characterised and will allow us to understand how wounds, which mimic those that develop in patients, respond to chemical application and how the barrier reforms. Genetically altered animals (for example diabetic animals) will be used to reflect the pathology of wounds in humans which tend to be particularly problematic in diabetic patients. Non-invasive imaging may be used to track the delivery and distribution of the administered therapeutics agents.

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

Genetically modified animals-Some of the animals will have an altered immune system making them more susceptible to infection. Extra steps will be taken to ensure these animals are prevent any infection and thereby minimising the likelihood of compromising health. Some animals will develop diabetes-like symptoms. Similar to humans, these animals may develop symptoms such us weight gain however tight measures are in place that will ensure the animals welfare is maintained.

Chemical barrier penetration - There would be no tangible adverse effects foreseen for most of the studies as the application of the devices e.g., the microneedles or suction device, is pain-free and does not cause irreversible damage to the tissue. The chemicals used in the studies have been well- characterized prior to use in live animals and thus their effects and potential side-effects are known.

Extraction of chemicals out of tissues – Skin stretching has been used previously in animals as well as in human participants in clinical studies at by the applicant and it is known to cause no damage to the skin when employed at specific pressures and times. The skin of the animals will be closely monitored for any redness.

Local inflammation models- The animals are expected to experience some discomfort and hence close monitoring will be in place to ensure that the animals are not in distress. To minimise any discomfort or suffering, only well-characterised doses will be used, extra bedding will be added to the cages, during the paw inflammation model, and the duration of the experiments will be kept at the minimum required to obtain the required information (typically 6 h).

Superficial Tumour models- The animals may experience moderate discomfort but close monitoring and fastidious process of maintaining welfare will be put in place to ensure there no excessive distress or pain.

Nail infection treatment- The topical infection of tissues with dermatophyte fungi has been frequently reported in the literature. The infection remains local and there are no reported incidences of invasion and dissemination. It is likely that both the nail and skin will become infected, but both only induce mild topical tissue distribution without pain. It is common for Guinea Pig's kept as pets to develop similar infections and for them to be reversed by topical application of antifungal agents. As the infection is on the outer surface of the body and it will be enclosed by a bandage the animal's general health and welfare as well as ability to move normally will be closely checked.

Wound healing studies- The animals may experience moderate discomfort and there is a risk of infection when damaging external biological barriers. However, wound models have been well described in the literature and close monitoring practices well developed including the use of suitable medication will be used to minimise any discomfort.

Several studies will be conducted under non-recovery general anaesthesia which will reduce the animal's distress or discomfort. Any adverse effects that may arise during these studies will occur under anaesthesia hence the animal will not experience any pain and if necessary, the animal will be culled before recovery. Whenever recovery is necessary, then multiple procedures (e.g. administration of medicines using injection, application of suction to the skin) will be completed under the same general anasthesia session, the session is kept to the minimum required (typically 30 min) and the animals are allowed to fully recover and will be closely monitored to ensure that natural behavior is regained between anasthesia sessions.

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 – up to 15 % of mice, rats and 10% guinea pigs
- Mild- up to 40 % of rat and mice and guinea pigs
- Moderate- up to 45 % of mice and rats and up to 50% the guinea pigs

What will happen to animals used in 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 important to achieve the objectives of this project as it allows the investigation and understanding the total body exposure of chemicals in a manner that translates to humans. The animals have been selected carefully for each section of the work based on their ability to replicate the topical biological barrier properties (e.g., skin, nail, inside of the mouth) in a similar manner to that of humans. Although in vitro and ex vivo work is very important and informative, it is limited as it does not t account for the blood flow or interstitial fluid flow, which both help to transport molecules (either biomarkers or therapeutic agents) through the topical biological barriers. Therefore, in vivo experiments are required to get a better understanding therapeutic agents and biomarkers movement across the barriers and facilitate the translations of the findings into the clinic. For example, the in vivo studies conducted previously under the current license has enabled the development of a novel non-invasive device (Device patent filed, Apr 2024) for the profiling of skin biomarkers to investigate various conditions such skin cancer, eczema and skin aging. This has led to a number of clinical studies, using this non-invasive device, working with healthy volunteers as well as those with diseases (such allergies and eczema) and would have a direct impact on the prevention, diagnosis and monitoring of health for both humans and animals. The ability to conduct clinical human studies, using such non-invasive devices, allows to reduce the number of animals required to complete the research studies objectives.

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

In silico computer simulation coupled with ex vivo studies, completed using excised tissues and cells have been used to predict and characterise the chemical transport, metabolism and biological effects. Microbiology studies have been also used to investigate the optimal dosing of agents prior to in vivo investigation. Other technologies (such as organ-on-a-chip) have been considered but were not found to be beneficial as these do not reflect the complex structure and barrier properties of the topical biological membranes.

Why were they not suitable?

In vitro work does not replicate the environment found in a living animal and the complex structures of the topical biological membranes (such as the skin) and do not account for the flow of the body fluids in the tissue (e.g. blood and interstitial fluid) which have an important role in molecules transport.

Furthermore, it is important to understand the immunological response to chemical absorption and this can only be fully understood 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 numbers have been estimated based on our previous work (computer modelling, ex vivo studies and previous in vivo studies)

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 to be used in the studies, various experimental studies are first conducted in silico, in vitro then ex vivo. This information enables us to design a refined study plan and tools such as NC3R's Experimental Design Assistant has been explored to achieve this aim. For example, different doses of applied chemicals are commonly explored in ex vivo tissues to establish what dose is required to achieve passage across the tissue, then this dose is used in the in vivo studies. In addition, ex vivo tissues have been used to establish if several of the chemicals have any toxic effects in the tissues, e.g. hydrogen sulphide has been applied to skin in the laboratory and histology has shown no local damage and no effect on the barrier properties.

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 conducting large in vivo studies, small well-designed pilot studies will be first conducted. These will be informed from studies completed in vitro and ex vivo. where appropriate computer modelling and simulation will be used to minimise the frequency of the in vivo studies and the number of animals involved.

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 porcine skin and other topical biological membranes (e.g. inside of the mouth) display a closer similarity to human topical membranes whereas rat skin for example is more permeable (approximately 11-fold more permeable than human skin (Roy et al., 1994). Despite such an increase in skin permeability rodent skin displays a similar anatomical structure (i.e. dermis, viable epidermis and stratum corneum as well as the presence of skin appendages) and functional properties to human skin. Although the magnitude of molecular transfer enhancement achieved may differ when using rodent and human skin, the pathways and mechanism by which molecular transfer occurs is anticipated to be similar. During this work, the therapeutic agent permeation and biomarker extraction will be investigated across the topical membranes using novel non-invasive methods using optimised approaches such as to cause no or minimal temporary disruption to the membrane (such as the skin) and using the shortest time possible to ensure an efficient outcome. These will be compared to traditional administration route using well-established and refines methods.

For the local inflammation studies, rats will be more suitable than mice as the small size of the mouse’s paw and ear does not allow local application of the chemical extraction device. The ability to apply the device directly onto the inflammation site enable us to

investigate chemical biomarkers extraction across the skin and allows the assessment and monitoring of therapeutic interventions. The protocol for the inflammation studies has been previously optimised (and will continue to be reviewed when necessary) to induce minimal pain or discomfort to the animals. For example, the acute inflammation model will only be used for a maximum of 6 h and is restricted to one local site. In addition, steps will be taken to enhance the welfare of the animals during this time. The low-grade inflammation model (similar to that obtained due to skin aging) has been optimised to cause minimal local inflammation in the skin (manifest by slight redness on a small area of the skin) and the level of inflammation caused is guided by measuring the level of disruption using a non-invasive probe.

Mice will be used for the cancer studies as the site of the superficial tumours (e.g. dorsal skin on the flanks) allow efficient application of the suction device. The melanoma model is well established in mice and the analytical kits that allow understanding of the effects of chemical absorption and chemical extraction are commercially available, which maximises the amount of information that can be generated in a single experiment. The method has been optimised in our previous work to ensure controlled tumour development and the welfare of the animals. Direct sampling of biomarkers from the tumour site has been shown to not affect the normal tumour development. Close monitoring of the mice will be in place to quickly detect and resolve any unexpected side effects, although these has not been present in previous work.

For studies involving the nail, rats will be used initially to investigate how the treatment permeate and affect the nail however, Guinea Pigs are required as they are the smallest animal that have nail tissue that is representative of humans (rabbits are an alternative but larger animal). The nail infection model has been established and published in this species and thus it reduces the number of animals needed for the work due to failure of the infection model.

For the wound healing studies, the diabetes and wound healing models to be used are well documented and have previously been demonstrated to be robust and reflect the wound progression in clinical scenarios in humans. The majority of the studies in this section will be performed in rats since mice are reputed to heal too quickly, at least in non-diabetics that we will use in some control studies.

Additional advantages of using rats over mice are the ease of handling, comparative ease of drug administration, and the quantity of tissue and other samples that can be recovered for later analysis. Close monitoring will ensure the animals will be maintained in good general welfare of the animals and would allow us to quickly detect and resolve any issues that may arise,

For all the studies where the use of a novel medical device in involved, the devices have been 3-D printed in house and have already been optimized to be non-invasive or minimally invasive, so these do not disturb the barrier properties (e.g. skin, nail and buccal membrane). The size and the design of the devices would continuously be reviewed with the ability to easily alter these characteristics to improve the fit, the comfort and performance of these devices.

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

Biological barriers need to be fully differentiated and represent elements of the human barriers for it to generate useful information. For this reason, adult rodents and whenever possible, adult mice will be used. Rats and guinea pigs will only be used when the study objectives strictly necessitate it.

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

All the animals involved in the studies will be closely monitored for welfare, normal behaviour and emergence of any adverse events over the time of the study. The scoring systems will be designed to be objective but will be adapted for each study and dependent on species and strain. With judicious use of this welfare scoring, early onset of any adverse events should be detected quickly and steps to be taken to alleviate any distress to the animals. The information is continuously reviewed and used to refine any further studies. In addition, testing on tissues ex vivo and running small pilot studies prior to the commencement of any new work allows us to minimise any likelihood of adverse events and causing any distress to the animals. When required, the animals will be treated with therapeutic agents (e.g. analgesics) to minimise any suffering or discomfort, unless this will interfere with the scientific objective and outcomes of the study.

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

- ASPA Code of Practice
- NC3Rs website and literature
- Nuffield Council on Bioethics (available on-line https://www.nuffieldbioethics.org/)
- The RepRefRed Society Website and documents https://www.reprefred.eu/Refinement
- Workman et al, Guidelines for the welfare and use of animals in cancer research. Br J Cancer 2010

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

This will be ensured by attending courses, provided by NC3Rs to engagement with the NTCO and unit NACWO as well as discussion with other researchers.

55. Inflammatory mechanisms of neuropathic pain and associated sensory changes

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

Chronic pain, Neuropathic pain, Inflammation, Nociceptors, Cannabinoids

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 aim of the project is to investigate how inflammation of a nerve may contribute to neuropathic pain. Specifically, it will aim to identify novel inflammatory and neuronal targets for treating neuropathic 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?

Neuropathic pain affects approximately ten percent of adults in the UK. It can be extremely debilitating and comes at substantial economic cost in terms of healthcare and lost workdays. Many patients describe symptoms such as shooting or burning pain, increased

sensitivity, as well as pain that radiates when limbs are moved. Work from our laboratory suggests that for some patients, nerves in the limbs or back are inflamed. These patients include those diagnosed with nonspecific back or arm pain, or radiculopathy. Such conditions are extremely common, but due to our lack of understanding of what is causing the pain, current treatments are often ineffective. Therefore, there is a need to better understand what is causing the pain and how to target the pain, so that more effective treatments can be developed.

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

The expected outputs are scientific publications that identify specific pharmacological targets for the treatment of neuropathic pain.

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

The short-term benefits are that this project will advance our understanding of the mechanisms of neuropathic pain. It will provide valuable information on possible drug targets for treating neuropathic pain. In the long-term, information gained from this study may be important for future drug development.

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

The outputs of this work will be maximised by publication of the data in scientific journals and presentation of the data at international scientific meetings. Furthermore, the data will be shared with our collaborators and stored on the university repository, which is open access. Data will also be presented to our clinical colleagues through meetings within the local NHS Trusts.

Species and numbers of animals expected to be used

- Mice: 200
- 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.

The project will use adult rats and mice. The models that will be used in this licence were developed on rats, and therefore the results can be compared to previous studies. Other laboratories have successfully induced similar inflammatory models in mice. Collecting data from mice will allow us to explore in future studies the use of genetically modified animals, such as knockout mice, to determine how switching off specific genes affects the development of neuropathic pain. Furthermore, rodents are the lowest vertebrate group on which these types of experiments can be carried out.

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

Animals will undergo surgery to inflame a nerve in the hindlimb. In other animals, the nerve will be treated with drugs to stop the movement of proteins along nerve fibres (also called axons). Surgery will involve wrapping the nerve with a small strip of absorbable sponge saturated in different agents.

Before and after surgery, the skin on the paw will be tested for changes in sensitivity to direct pressure, heat and cold by applying different stimuli. In some animals, drugs to treat the inflammation or reduce the paw sensitivity will be injected into the abdomen, veins, muscle or under the skin. In other animals, these drugs will be topically applied to the nerve during the surgery to inflame the nerve or disrupt the movement of proteins. Finally, animals will be deeply anaesthetised, and the function of nerve fibres that carry pain information will be examined by recording nerve impulses in response to electrical stimulation or the application of pressure, heat, cold or chemicals to the nerve. Most animals will be examined within two weeks following the initial surgery. At the end of each experiment, animals will be humanely killed.

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

The surgery to inflame a nerve, or stop the movement of proteins, does not cause significant adverse effects. Occasionally, the skin incision can cause minor discomfort, and although unlikely, infection can occur at the surgery site. Significant damage to the nerve during surgery is rare but can affect how an animal moves. Testing the sensitivity of the paw to pressure or different temperatures triggers a brisk reflex, whereby animals rapidly lift and then immediately lower their paw. Also, it can occasionally cause temporary discomfort, which only lasts a short duration. We do not anticipate the injection of drugs to treat the inflammation or reduce paw sensitivity to cause adverse effects. The surgery is performed with minimal damage to tissues, such as muscle and nerve, and based on our experience, animals do not show obvious signs that they are suffering from pain. However, immediately following the surgery, animals will be given an analgesic to stop any pain, and a local anaesthetic will also be infused around the surgical site to prevent discomfort from the procedure. All animals will be monitored closely, and if an animal develops obvious illness or signs of pain, it will be used for terminal electrophysiology or humanly 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)?

Approximately 90% of animals will experience moderate severity. Approximately ten percent of animals will not undergo surgery and will only be administered control test substances that do not cause adverse effects. These animals will experience only mild severity.

What will happen to animals used in 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 mechanisms of neuropathic pain involve multiple systems within the body, which include the immune, circulatory, and the peripheral and central nervous systems. Due to their complexity, it is not possible to study the effects of inflammation on these systems, or how they interact with one another, using non-animal alternatives.

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

We have considered both cell culture and computer models as non-animal alternatives. However, having reviewed the scientific literature, neither of these can be used to achieve the aims of this project.

Why were they not suitable?

Cell culture would not allow us to examine the interactions between different systems, such as the immune, circulatory, and nervous systems. Therefore, we could not achieve the study aims. Similarly, computer models are not effective for studying complex pain processes, because they are not able to model all components of the pathway, many of which are unknown.

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 our published studies, as well as usage on our previous project licences. Animal numbers will be kept to the minimum number that are required to show valid effects. Typically, group sizes will be approximately ten animals. In most experiments, the animal model will be compared to groups of animals that have undergone a sham surgery, which will enable us to control for the effects of surgery. However, it may sometimes be possible to compare to previously published data, or to animals that have not received surgery. Animals receiving a test drug to treat the inflammation or reduce the paw sensitivity (e.g., a cannabinoid), will be compared to animals that have received the vehicle for that drug (i.e., the substance used to deliver the drug). This will enable us to determine whether any physiological effects, such as changes in paw sensitivity or spontaneous firing of nerve cells, are due to the drug alone. For new drugs that have not previously been tested in animals, a pilot study will be performed on a small number of animals (approximately three) following surgery where the drug will initially be administered at a low dose. The dose will be increased in subsequent animals until an effective dose is established that prevents pain behaviours in those animals.

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

The experimental design has been discussed with a statistician. A Power calculation has been performed to determine the smallest number of animals that are required to produce a meaningful result. However, before commencing individual studies under this licence, the NC3Rs design assistant will be used to establish the optimum 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?

Our laboratory has substantial experience at using these models and techniques and has published extensively in this field. As such, we will ensure that our protocols will use the minimum number of animals. In addition, the experiments will be discussed with our collaborators, and other experts in the field, prior to commencing the work to ensure the optimum number of animals will be used to achieve our aims. Drug doses will be determined from previously published data or from data obtained by our 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.

Most studies examining the mechanisms of chronic or neuropathic pain use animal models that involve traumatic nerve injury, which can lead to significant welfare issues due to the extensive surgery and injury to muscles and nerves. However, we plan to use a less severe model that does not cause significant injury to nerves. Local inflammation of nerves, and disruption of transport along nerve fibres, is not associated with the loss (degeneration) of nerve fibres. Animals recover after two weeks, and there are no obvious adverse effects. The application of direct stimuli (such as pressure, heat and cold) to the paw to examine changes in the sensitivity to pain only cause transient discomfort. In addition, we will explore the use of novel methods of assessing pain that examine more natural behaviours that do no cause distress or discomfort. For example, we plan to assess how our animals burrow, as well as their facial expressions. Rats are currently the lowest vertebrate group on which our models have been successfully performed.

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

Less sentient animals could not be used because their immune and nervous systems are too different from human equivalent systems. Also, behavioural measures of pain could not easily be performed in less sentient animals.

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

Environmental enrichment, such as tubes and wooden chew bars, will be used in all cages, and animals will be housed in groups. Soft nesting material will always be used. Animals will be handled regularly by the study team and animal technicians. Animals will be weighed regularly, and grooming behaviours and gait recorded. Grimacing will also be examined as an indicator of pain and distress. A pain score developed with the named veterinary surgeon (NVS) and the named animal care and welfare officer (NACWO) will be completed regularly for each animal. To reduce stress, animals will be habituated to the behavioural testing apparatus, and trained prior to testing pain behaviours.

Postoperative care will include placing cages on heated mats and close monitoring of animals. Analgesia will be used such that it does not interfere with the experiment.

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

All work will abide to both local and published guidelines (e.g., NC3Rs, IASP, and ARRIVE guidelines) to ensure best practice is followed. We will use the following publications and websites to ensure our experiments are conducted in the most refined way:

Arras et al., Assessment of post-laparotomy pain in laboratory mice by telemetric recording of heart rate and heart rate variability. BMC Vet Res. 2007; 3: 16.

Hawkins P. Recognizing and assessing pain, suffering and distress in laboratory animals: a survey of current practice in the UK with recommendations. Lab Anim. 2002 Oct;36(4):378-95.

IASP Guidelines for the Use of Animals in Research. https://www.iasp-pain.org/resources/guidelines/iasp-guidelines-for-the-use-of-animals-in-research/

IASP Animal Models for Translational Pain Research. https://www.iasp-pain.org/resources/fact- sheets/animal-models-for-translational-pain-research/

Rat Grimace Scale. https://www.nc3rs.org.uk/3rs-resources/grimace-scales/grimace-scale-rat.

Recognition and Alleviation of Pain in Laboratory Animals. National Research Council (US) Committee on Recognition and Alleviation of Pain in Laboratory Animals. Washington (DC): National Academies Press (US); 2009.

Sotocinal SG. The Rat Grimace Scale: a partially automated method for quantifying pain in the laboratory rat via facial expressions. Mol Pain. 2011 Jul 29;7:55.

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 by regularly checking for updates on the NC3Rs website and reading the NC3Rs newsletters. We will also attend relevant NC3Rs webinars.

Furthermore, we are kept informed of updates by the Named Information Officer at our institution. Experiments will be redesigned based on advances in the 3Rs.

56. Pharmacokinetic and pharmacodynamic studies to support Neuroscience 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, Pharmacodynamics, Neuroscience, Pain, Neurodegeneration

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 main aims of the licence are to

- Enable us to decide what doses of potential new treatments we need to use in our neuroscience disease (conditions affecting the nervous system) models. We will do this by dosing animals and taking blood samples to measure how much of the treatment is present at specified times after dosing, which is termed as pharmacokinetics (PK).

- Enable us to understand how much of the treatment is needed to treat the disease. We will do this by dosing animals which have had a pre-treatment or challenge (for example, a substance such as a protein is administered to mimic all or some aspects of the disease), so that we can measure both the amount of the challenge in the blood (PK) and the effect of the challenge or potential novel treatment on the body (pharmacodynamics - PD). Both pieces of information may be used to build a mathematical PK-PD 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 a constant need to discover and develop new treatments for neurological diseases such as (but not limited to) pain (for example – migraine, arthritis, neuropathies etc) and neurodegeneration (for example – Alzheimer's, Parkinson's, Huntington's etc). To bring these life changing medicines to patients takes many years of research and requires us to show that these treatments are effective and safe in humans. 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 or animal cells/tissues, how it interacts with tissues from patients, how it behaves in relevant animal models of disease and how much we will need to give to patients for it to be effective.

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

Work performed under 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 and how much of the treatment needs to be given to be effective. For example, we will obtain an understanding of the maximum concentration of the treatment in the blood, which parts of the body it reaches and how long it takes to be eliminated from the body. This information is required by our research teams to plan and deliver further studies to look at how effective they are.

These findings, along with information from human and animal cell (in vitro) experiments, will enable us to move suitable novel treatments into human clinical trials. Ultimately, these will result in new medicines to treat patients. Most data generated from these studies are often reported when we publish results in disease models.

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

As our focus is on delivering effective medicines to patients, the work performed under this licence will contribute to the overall goal by generating specific information that is used to determine how (if the medicine needs to be dosed by mouth or by injection) and how much (what the dose or volume given may be) of the treatment needs to be given to have an effect. Without the understanding of how much or how often (for example - if it needs to be given once or twice daily) to give, we cannot conduct effective research in animal models of disease or predict how much we may need to give patients.

These studies will be used to support specific research into new treatments for neurological diseases that affect many patients and have life limiting impacts.

Developing potential new treatments 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 in the previous years are now in the clinical testing phase and we are confident that this will continue in the future years.

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

We have an open culture where interactions and collaborations between our scientists and those from other research organisations are encouraged. Whenever possible we regularly

share our findings at academic conferences and symposia. However, due to the commercially sensitive nature (termed as intellectual property) of our work, we are unable to be open about our very early and novel findings until appropriate patent protection is obtained. We may also consider disseminating unsuccessful approaches either through publications or platforms such as F1000 research, where possible and not limited by intellectual property.

However, 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 (Replacement, Reduction, and Refinement of animal research).

Species and numbers of animals expected to be used

- Mice: 3000
- 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.

The majority of these studies will be performed in adult mice. Mice are often used to model human diseases as they are a mammalian species with a similar (but not identical) organ anatomy and physiological system as compared to humans. Often in efficacy or PD studies, we either use genetic modifications or administer substances that can induce disease in adult mice. Although neurological diseases can affect patients at any age, the majority of rodent studies are performed in adult animals 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 better established in the rat, or there is some other technical need (for example – if the gene or protein in the rat is closer to the human in its properties, than those from a mouse).

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

For all studies, animals will be allowed to acclimatise for 7 days in their home cages after arrival into the unit. In a majority of cases (unless it interferes with the welfare or scientific outcomes), animals will be group housed, have easy access to food, water and provided enrichments (such as wooden blocks or balls, tunnels, cardboard homes etc). After the acclimatisation period, often animals are microchipped for identification purposes. Following this, the animals will undergo limited handling for another 7 days to help recover from the implantation.

To allow for novel treatment to be administered to animals, we will need to ensure that the doses of the treatments are safe and well tolerated. To support this, we may run tolerability studies to derisk and identify any concerns with the novel treatment. These studies will involve dosing the novel treatments in a staged manner (usually from a low to high dose) to ensure the treatment is well tolerated and does not adversely impact the animals.

In a typical PK study, animals will be implanted with identification microchips under anaesthesia and a few days later weighed and dosed with the novel treatment. The compound may be administered using a range of techniques (such as through the mouth

or injected), which will closely mimic what is anticipated to be used in the clinic. Often, these novel treatments may also be administered on multiple occasions, to identify what the most effective way to dose in humans would be. As our focus is on neuroscience, we may also at times dose into the spinal canal or brain directly, under anaesthesia.

Animals are returned to their home cages after dosing and then at certain time points (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 (usually from the tail vein) using a small 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 needle is placed directly into the heart to take a large volume of blood, followed immediately by killing the animal. Deep anaesthesia will be terminal (at the end of life) and the animals will not regain consciousness before being killed. Apart from these typical experiments we may occasionally dose using surgical implantation of a delivery pump under the skin. This allows for the treatment to be released in a controlled manner without the need to administer multiple times, which may cause distress to the animal.

Some studies (typically PD studies) may also include giving the animal a challenge or insult (such as an administration of a substance that can mimic some or all aspects of a disease) so we can understand the effect as well as exposure of the novel treatment (PK-PD effects). These may look similar to a PK study but with the additional injection of the challenge. Since we will be looking at the effect of both the challenge and the novel treatment, we may also use readouts such as physiological (such as temperature ad blood pressure recording) or behavioural assessments (such as those to measure pain) and imaging, to better understand the effects of the treatments.

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

Administration of novel treatments may cause some brief discomfort during or immediately after dosing. This is transient (short-lived) in nature and animals are not expected to show any signs of ongoing discomfort. Similarly, blood sampling is likely to result in transient pain or discomfort and should not have a lasting effect. Direct administration of novel treatments into the spinal canal or brain will be done under anaesthesia. Sometimes, we may use surgical implantation of delivery systems to administer treatments. These procedures will be followed by additional monitoring after administration to ensure smooth recovery after the procedure and also due to the treatment given.

The treatments we will dose are not expected to result in any adverse events. For novel compounds that have not been tested in animals before, we will run a small trial study (tolerability test) to ensure it is safe to be administered. In studies where we will use a challenge, some animals may exhibit clinical signs such as reduced grooming, reduced social interaction and lowered body temperature.

Additionally, they may also cause some clinical signs seen in neurological diseases such as pain and sedation. These reactions are expected to appear and are not known to cause any harm apart from transient discomfort.

Physiological and behavioural measurements used are not expected to produce any adverse effects other than those that are transient and a consequence of handling or applying a stimulus to measure a response. For example, dosing with lipopolysaccharide (stimulus), can result in increase in cytokines (substances that can control the activity of blood and immune cells) that can be measured in the blood. The behavioural endpoints

used to measure responses to mechanical (such as touch, pressure) and thermal (such as heat and cold) stimuli are not expected to cause any harm. To further refine the process, we have specified cut off points to prevent any injury or tissue damage due to testing.

Although a majority of the procedures performed only cause transient discomfort, the collective effect of it all can have a slight long-lasting effect, and hence the animals may experience moderate severity of discomfort overall. If any adverse effects beyond those expected occurs and welfare concerns are anticipated, the Named Veterinary Surgeon (NVS) and Named Animal Care and Welfare Officer (NACWO) will be consulted. Any severe adverse effects resulting in an alteration of appearance, weight loss, food and water intake or natural behaviour will result in the animal being 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 ~ 50%

Moderate ~ 50% Rats:

Mild ~ 50%

Moderate ~ 50%

These proportions are estimates and may change in either direction depending on the number of studies required to be run in each of the protocols.

What will happen to animals used in 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 possible to accurately predict the PK properties of novel treatments using non-animal alternatives, so animal studies are very essential. Much effort is put into screening out unsuitable compounds during the early stages of research. For example - by using human and rodent liver cells in vitro, we can test how fast the compounds may be broken down and using other in vitro tests we can 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. 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.

Before testing a new treatment in an animal model we need to know how much and how often to give to have an effect. This licence will enable us to test new treatments in rodents and predict how much to use in rodent models. This information will ultimately allow us to evaluate the doses that we need to deliver to be effective in patients.

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

There are multiple stages of non-animal screening of new treatment, which are in place and performed before testing in animals. Depending on the nature of the compound, we will perform tests (both using computer modelling and laboratory testing) to understand the properties which can affect treatment 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 compound. These assays will also help us predict what doses we may need to consider dosing in animals. It is also impossible to assess the safety of a treatment without testing in animals. This is similar to human clinical trials where Phase I trials are often conducted to assess the safety of the treatment in healthy humans.

Non-animal alternatives such as in vitro testing in neurons (nerve cells, which are the most commonly studied cell types in neuroscience research) have been extensively used by many groups and show that a proportion of the fundamental properties are lost during growing (termed as culturing) these cells. This unfortunately can lead to misinterpretation of results. For example, in vitro cell culture systems have limited utility in informing us about pain perception, which can only be determined by behavioural studies (studying responses to pain in animals). Similarly, the degeneration (damage to cells) induced in cultures may not accurately reflect what happens in the body. Often, retaining just one of the fundamental properties is insufficient to inform us about the prevention of disease due to the complexity of the systems involved in the disease pathology. In addition, testing in culture systems alone cannot provide information about how effective treatments are, or the severity of disease, which is crucial to model and determine doses that are to be assessed clinically. The other non-animal alternatives such as organ-on-chip technologies have been gaining traction. This involves the development of tissue models in vitro that can closely mimic certain changes that occur in diseases and enable us to test the effect of new treatments. Although they do a particularly good job of being able to study relationships between different cells/tissues, they are unable to fully recreate the entire physiological environment, which may lead to misleading results, and ultimately the development of either an unsafe or ineffective compound for human use.

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 and cells, which could affect the way the treatment moves around, interacts with, is processed, broken down and eliminated from the body.

As these treatments are going to later be tested in animal models of disease, it is very important that we select doses (how much, how often and how to dose) which are likely to give us the required information and effects. These studies, more often than not, will also be used to guide the selection of doses for human clinical trials when combined with other information such as the way human cells or tissue systems interact with the new treatment.

Diseases affecting the nervous system are highly complex, requiring input from many parts of the nervous system. For this reason, in vitro testing alone is not enough to determine if new treatments will be effective. Furthermore, the use of animals will help determine if

these treatments are effective, which is impossible to deduce from in vitro systems. No currently available in vitro systems can fully replicate human physiology, as stated above. We fully acknowledged their strengths, are aware of and appreciate their limitations as these studies may be valuable but cannot completely replace what animal studies can tell us.

The physiological and behavioural measures that we use have been carefully refined to reflect what is seen clinically in human disease and will be chosen based on the scientific outcome expected. The amount of information we can obtain from carefully designed animal studies is greater and more valuable in translating to clinical studies in its impact than in vitro studies, as they cannot represent entire physiological systems. In addition, we will use human clinical data and mathematical modelling, where available, to predict what may happen.

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 have been based on current and previous usage (how many animals per study, how many studies of each type per year) and allowing for expected changes in demand.

All experimental designs will be reviewed often to ensure appropriate treatment and control groups are used to maximise scientific output and keep the sample size to a minimum. Where necessary, pilot studies will be undertaken using the least number of animals required. As we expect most of the data generated to be quantitative in nature, relevant statistical tests will be used to analyse all datasets.

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

When we run tolerability studies, where possible, we will aim to collect blood samples to look at PK profiles of the compound and avoid the need for running separate PK studies as a follow up. This will reduce the number of animals required to run 2 separate studies. Where data with different vehicles are available, we can limit using vehicle or controls groups in subsequent studies.

Generally with tolerability and PK studies, we are only trying to ensure that the treatment is safe, and it reaches where we want it to in the body. We may also want to look at differences in blood levels and how it changes with changes in the dose. We may also be 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 treatment to give and how often to dose). The number of animals per study (normally 2-3 per group) is therefore dependent on how many blood or tissue samples are required, for how long, and the number of samples required at each time-point.

We generally take more than one sample per animal (the limiting factor is blood volume as we do not want to impact animal welfare by sampling too much), so our analysis benefits

from multiple samples taken from the same animals. This will also help us follow up on the same animals and what is happening to them or the treatment in their body. Standard procedures normally require only 3 samples per time point, although this may be increased in certain situations. For example, when PK-PD studies are performed, as statistical methods are used in these studies. For some types of new therapeutics we may be able to dose a combination of different compounds and measure them all simultaneously, which further reduces the number of animals used. This, however, cannot be done as a standard method as there is a risk of interactions or technical limitations on the measurement technique.

In case of PD studies, as we expect most of the data generated to be quantitative in nature, relevant statistical tests will be used to analyse all datasets.

For a majority of studies that will be performed under the remit of this licence, we have included a weight loss limit of 20%. This will allow us to monitor any acute weight losses as from our experience, some compounds result in a brief weight loss, with the animals recovering within 24-48 hours. Having this weight loss limit enables us to prevent animal loss and eventually reduce the number of animals used.

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 increases the effectiveness of production and animal usage. Occasionally (for the PD studies), we may use genetically altered animals bred for scientific use and the numbers are carefully monitored to ensure overproduction and wastage is minimised. New treatments, which have not been tested in animals before, undergo tolerability studies to ensure it is safe to be administered into animals. This generally uses a low number of animals (2-3 per group) to confirm that these compounds 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 that a treatment causes an adverse effect that would stop the experiment. For most PK studies, clinical dose predictions are made based on what is observed in rodents.

In case of studies that require an optimal number of animals to generate robust scientific evidence, our group has adopted a Good Statistical Practice policy, under which all study designs are reviewed frequently by a qualified statistician. These designs are regularly reviewed to ensure best practices.

For procedural methods, we will also utilise online tools such as the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines, ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines form: a guideline basis for the determination of experimental design (https://arriveguidelines.org/) and the NC3R's (The National Centre for the Replacement, Refinement and Reduction of Animals in Research) experimental design assistant (https://www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda). This will ensure we maximise the scientific outcome of our work while using the least number of animals and that the correct analysis is carried out to give the best possible interpretation of the 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.

PK studies normally consist of dosing animals with the treatment, followed by taking blood samples, usually from the tail vein. Different dosing routes may be required for different types of novel treatments, depending on what is going to be used in the clinic or where in the body we want the treatment to reach. Sometimes we may need to deliver a constant amount of treatment over a period of time, and in these cases, we may use specialised small pumps placed under the skin surgically. This will also avoid the need to give multiple injections.

All procedures (especially the physiology and behavioural testing) have been refined to cause the least distress, whilst giving us the most beneficial scientific output. However, care will be taken to ensure the animals will be monitored and humane endpoints for when the animal needs to be killed to limit suffering have been clearly defined. For example, if we anticipate that the animal may lose weight (such as either due to administration of a compound or due to a procedure) during the study, we will weigh daily and humanely kill any animal that has a weight loss of 15-20% from baseline, depending on the type of study.

Particularly in case of the PK-PD studies, due to the chronic (long duration) nature of the most neurological conditions in the clinic, it is necessary to follow the animals for a few weeks to replicate the human disease processes.

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. This is predominantly due to the fact that the physiological system of these animals is not similar to that in humans.

Using animals terminally anaesthetised only would be hindered by limited duration and risk giving information that is compromised. For example, blood flow to the liver and other organs may be different under anaesthesia and could affect the measurements. Additionally, the presence of anaesthetic could alter the way material is processed by the body. This is also the case with the PK-PD studies that require behavioural and physiological measurements, that cannot be performed in less sentient or anaesthetised animals.

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

None of the procedures that will be performed under this licence are known to cause any lasting distress to the animals. For example, food and water intake are normal, animals show normal growth curves, and the general observed health and behaviour of the animals are unaffected by the new treatments. Measures to reduce animal distress will be undertaken throughout the study period. All animals will be allowed to acclimatise to the study environment and operator before performing any procedures. In most cases,

animals will be socially housed in groups, except for when there is a scientific or welfare need to singly house them (such as after surgical implantation of delivery devices where cohousing may possess the risk of harm due to interaction with other animals).

All animals will be checked daily and weighed at least once a week, irrespective of the nature of the study. Based on the techniques employed, additional monitoring, weighing and health checks will be considered to ensure adequate recovery from any dosing, procedure or surgery, and adherence to humane endpoints. If we employ surgical (for example, in case of delivery devices) or other invasive techniques (injections into the brain or spinal canal), we will provide peri-operative analgesia to ensure smooth recovery from the procedure.

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 during the study. Small needles (used for dosing) are always discarded after a single injection so that blunted needles (which can cause unnecessary tissue injury and pain) are not used. The tail vein is generally used for sampling blood. However, we can also employ alternate sampling procedures such as sampling from the saphenous vein (vein in the leg).

When using a dose of the compound which hasn't been tested before, we will run tolerability studies to ensure no adverse effects are expected. Where possible, we can combine tolerability/PK studies and PK/PD studies to avoid the need for multiple studies.

The compounds dosed or testing used in the PK-PD studies will have or already have been assessed to be the minimum which can be used to cause a significant reproducible biological effect without causing excess adverse events. Limits such as those for the number of tests and duration of exposure of the stimulus have been set out and will be adhered to, to avoid additional distress to the animals.

For example, if we are testing the tolerance of an animal to cold temperatures, the minimum temperature we will test is 0oC and the animal will not be tested for more than 3 days in a week to allow them to recover between testing and prevent any distress or tissue damage.

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

As a Project Licence holder I am engaged with local and national 3Rs groups and events and am kept informed by our Named Information Officer of relevant new information.

We will use the resources available such as the guidance and publications from the NC3Rs, PREPARE, AALAAC and LASA to inform us of any refinements that can be utilised in our studies.

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

All efforts will be made to follow the 3Rs throughout the course of this licence by learning from previous experience and any new studies we may run. We have signed up for the National Centre for the Replacement, Refinement and Reduction of animals in research (NC3Rs) newsletter and will regularly check for latest updates on their website.

Where possible, we will also attend any conference or symposia conducted by them and use alternatives or refined techniques. Regular consultations on the latest practical guidance from the Laboratory Animal Science Association (LASA), the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), the Institute of Animal Technology (IAT), and the

Royal Society for the Prevention of Cruelty to Animals (RSPCA) will provide additional sources of new recommendations.

57. Immune responses to viral infection and vaccines

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

Viruses, Influenza, Vaccine, Immunity

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?

This project aims to understand how the immune system responds to influenza infection and vaccination, and to use this information to develop better 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?

Seasonal influenza is estimated to cause between 1-4 million cases of severe illness and 300,000 to 650,000 deaths each year. Influenza pandemics have also occurred four times in the past 100 years, resulting in 50-100 million deaths. The possibility of a new pandemic remains of concern.

The best way to protect against influenza is through vaccination. However, due to antigenic change, influenza vaccine efficacy is frequently very low. We have identified regions of the influenza virus that appear to be restricted in their antigenic change but also highly immunogenic, making them ideal vaccine targets. By targeting these regions

through vaccination we hope to make better influenza vaccines and also understand how viruses, like influenza, evolve to escape immunity and/or cause pandemics.

Influenza is also used as a model viral pathogen. Consequently, the knowledge generated during the course of the project will help to increase our understanding of other viral pathogens (e.g. SARS-CoV- 2, norovirus, flaviviruses, respiratory syncytial virus (RSV) and rhinovirus) and help us develop better vaccines against them as well.

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

By the end of the project, we will have:

- Identified new vaccine targets.
- Developed a new single-dose vaccine that protects against all influenza strains.
- Determined the impact of prior immunity on vaccine efficacy.

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

Vaccine development.
Influenza causes between 1-4 million severe illness cases and 350,000-650,000 deaths worldwide each year. Often this disease burden falls on the very young or elderly, especially in low and middle-income countries (LMIC). In addition to this, 80 billion USD is estimated by the World Health Organisation to be lost due to influenza illness each year.By the end of this project, we aim to have developed a single-dose broadly protective influenza vaccine that will substantially help reduce the burden of influenza when compared to currently available vaccines.

Public Health.
The knowledge generated by this project will enable us to better understand the transmissibility and pathogenesis of influenza strains, which will help us improve public health measures to combat the spread of influenza. We expect that the work from this project will also progress into human clinical trials, eventually leading the vaccine(s) developed in this project to be widely available to the general public.

Influenza as a model virus.
Influenza is used as a model viral pathogen. The knowledge generated during the course of the project will help to increase our understanding of other viral pathogens (e.g. SARS-CoV-2, norovirus, flaviviruses, respiratory syncytial virus (RSV), and rhinovirus) and help us develop better vaccines against them as well.

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

All research using animals will be published in open access journals with wide readership. Data from the project will also be presented at international and national conferences. Best practice will be shared at these events and through publication.

We expect that the work from this project will also progress into human clinical trials, eventually leading to the vaccines developed in this study to being widely available to the general 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.

To fully study the immunogenicity and protective efficacy of mammalian vaccines there is no adequate alternative but to employ animals. The immune response to vaccination and infectious disease involves multiple, complex systems interacting in a physiological environment which cannot be replicated in tissue culture as there are currently no *in vitro* predictors of *in vivo* immunogenicity.

In this project, we propose to use adult mice to assess the immunogenicity of vaccines and immune responses to viral infection. Mice are the organism typically used to assess the immune response to vaccines or viral infection for pathogens such as influenza.

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

Mice may undergo *intra muscular* immunisation (AB) with a single or combination of (i) a protein- based, (ii) a protein-RNA/DNA, (iii) RNA or DNA-based (iv) attenuated influenza virus, (v) adenovirus or (vi) polymer nanoparticle-based vaccines, one to three times at intervals not shorter than 2 weeks.

Vaccination will occur in the same leg to stimulate affinity maturation in the same germinal centres and induce the best possible immune responses (*Vaccines* 2021 18;9(1):61).

One week after immunisation, blood samples may be collected via a superficial vein (e.g. tail, or saphenous) using standard guidelines. Blood sampling will consist of no more than 10% total blood volume in 24 hours, and no more than 15% in 28 days. In the unlikely event of the blood sampling site failing to clot spontaneously, pressure will be applied to the site to stop bleeding.

Before or after immunisation, mice may be challenged with less pathogenic influenza A or influenza B via *intra nasal* administration of an infectious dose (AB). Influenza doses will be given that produces a 15% or less weight loss.

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

Intra muscular immunisation (AB) is likely to produce short-term/transient soreness and fatigue. Blood sampling is likely to produce short-term/transient soreness and fatigue.

Intra nasal influenza challenge (AB) is likely to induce weight loss, reduced movement and fatigue for 5-7 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)?

Mice immunised by *intra muscular* immunisation (AB) are likely to experience mild severity.

Mice bled via a superficial vein such (e.g. tail or saphenous) are likely to experience mild severity. Mice challenged with influenza (AB) are likely to experience moderate severity.

The severity of the procedures will to tracked and recorded to ensure that the accumulated procedures (immunisation, tail bleeding and in some instances challenge) will not collectively exceed moderate.

In total, 25% of mice are expected to reach moderate severity due to influenza challenge, whilst 75% of mice are expected to reach mild severity due to experiencing immunisation only.

What will happen to animals used in 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 immunogenicity and protective efficacy of mammalian vaccines there is no adequate alternative but to employ animals. The immune response to vaccination and infectious disease involves multiple, complex systems interacting in a physiological environment which cannot be replicated in tissue culture as there are currently no *in vitro* predictors of *in vivo* immunogenicity.

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

At each point in the development of our vaccines we will ensure that they are robustly designed and fully assessed prior to moving them into *in vivo* studies. Such assessments involve (i) computational design using modelling software, (ii) serological assays using monoclonal antibodies and/or human sera to determine vaccine reactivity, as well as studies using purified human peripheral blood mononuclear cells (PBMCs) to test for B-cell reactivity in various human cohorts.

We will continue to follow the literature and adopt any improved assessment strategies during the course of the licence.

Why were they not suitable?

Due to the complexity of the immune system, as well as regulatory requirements for clinical trials, none of these *in vitro* systems are adequate to fully assess the immunogenicity and protection provided by a vaccine candidate *in vivo*. We will continue to use them to develop our vaccine prior to using them in *in vivo* studies, which are only proceeded with when all other assessment strategies have been exhausted and have provided a positive indication that a vaccine candidate will be successful.

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 published work by ourselves and others studying influenza vaccines, we can assess the numbers of mice required. We estimate up to 200 mice per PIL holder will be used per year, leading to approximately 400 mice used per annum (*Nat Commun.* 2018 9(1):3859; *Front Immunol.*
2018; 9: 126).

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

Pilot studies and prior published data are used in power calculations to assess the minimum number of mice required per experiment (in consultation with the departmental statistician). Past experience enables selection of experimental time points that maximise the amount of data while using the minimum number of time points. We will continuously evaluate and update our statistical approaches and group sizes. Based on our previous experience, a group size of 5 is typically associated with >80% power for the outputs required (*Front Immunol* 2018 31;9:126).

Where possible the data from each individual animal will be maximised through the collection of multiple tissue samples at endpoints and/or sequential sampling from the same animals across a time course, e.g. blood sampling.

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 used small scale pilot studies consisting of 1-3 mice to test the safety and impact of immunogens and challenge viruses.

Furthermore, we will use computer modelling of vaccine responses and power calculations based on previously published studies and pilot study data to determine mouse number (e.g. *Front Immunol* 2018 31;9:126).

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 adult mice for these studies since they are the most characterised

species for detailed immunological analysis and have well characterised disease courses for influenza.

Mice have proved to be excellent indicators of immunogenicity enabling the clear assessment of novel vaccines and vaccination regimes for improvements. Because of the numerous different immunological tools available, adult mice can be used for detailed characterisation which is not possible in higher organisms.

We will use our previous experience of vaccine dosing as well as published data to determine the minimal doses, smallest gage of needle, and vaccine volumes required to induce immune responses, and also leave mice to recover between immunisations which mice being left to recovered for at least two weeks.

We will use our previous experience of influenza challenge as well as doses provided via the published literature (*Front Immunol* 2018 31;9:126) to enable us to minimise the severity of the challenge and accompanying weight loss to <15%.

Most animals in our experience will show no clinical signs in the majority of experiments.

We have included a moderate severity to incorporate the possibility that with new vaccine/challenge versions or batches, some signs could be seen initially. If that is the case we will modify the protocols as done previously to reach the same minimal level of impact on the mice.

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

As adult mice are the most studied model animal for vaccine studies, they are therefore the most suitable model animal to use. Mice will be anaesthetised during *intra muscular* immunisation (AB) and *intra nasal* viral challenge (AB).

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

Past experience enables selection of sampling time points to maximise the value of the data while minimising the number of time points. For example, blood samples will be done only at the most informative time points post-vaccination.

For Influenza challenges, less pathogenic influenza A and B strains, such as H3N2 X31 or H1N1 PR8, and modified infectious doses that do not cause such severe weight loss will be used whenever possible. To more stringently assess the ability of new vaccines to protect against all sub-types it may be necessary to test efficacy with more virulent strains of influenza. Only vaccines that demonstrate robust cross-subtype protection against less pathogenic strains will be subsequently tested against more virulent influenza viruses.

To minimise distress, typically mice will be allowed to recover for at least 1 week between procedures and as well as weighing to monitor weight loss. Furthermore, up to two repeat immunisations will take place at least 14 days after the first immunisation using the smallest possible gage of needle to assist with healing and minimise discomfort.

Handling of mice will involve 'cupping' to minimise stress as per NC3R guidelines.

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

We will check the NC3Rs website on a regular basis to check for any refinements that could be applied to our studies. We will use the NC3Rs 3Rs self assessment tools to evaluate our activities and work on the feedback we receive.

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

There are many advances being made in the 3Rs and in order to stay up to date we will regularly review the NC3Rs website where information of latest advances can be found for specific techniques. In addition, we have regular updates regarding various advances from our NIO and NACWO.

58. Molecular mechanisms of cardiovascular 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

Key words

Cardiovascular homeostasis, Cardiovascular disease, Pregnancy complication, Antioxidant genes, Angiogenesis

Animal types	Life stages
Mice	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?

The aims of the project are to understand the underlying mechanisms of cardiovascular disorders, including preeclampsia and atherosclerosis. We will investigate the roles of the key protective pathways in the pathogenesis of these conditions and the therapeutic potentials of theses pathways for cardiovascular 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?

Despite the progress made in cardiovascular disease (CVD) research and management, CVD remains the leading cause of mortality and morbidity in men and women worldwide. Recently, the decline in the CVD related mortality rates has slowed down. In England, premature death rate for cardiovascular disease has risen year-on-year since 2020. The

economic burden for CVD related care in the UK is estimated to be around 25 billion annually. This is largely due to the lagging in cardiovascular drug and diagnostic innovation.

Hypertensive pregnancy disorder, such as preeclampsia, is considered as vascular disorder. It is also a risk factor for CVD. Both the mother and the offspring are at increased risk of developing CVD including hypertension, heart disease and diabetes later in life. It is not fully understood whether the etiologic pathways of pregnancy complications are associated with CVD. This project will enhance our understanding of the pathogenesis of these conditions and provide the rationale and foundations for developing novel therapies for the treatment of preeclampsia, an unmet clinical need, and other CVDs.

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

We will use mouse models of different cardiovascular disorders, including genetically modified mouse models, to identify novel molecular pathways that are involved in the pathogenesis of cardiovascular disorders, such as preeclampsia and atherosclerosis and peripheral vascular diseases. We will have a better understanding on why women have had preeclampsia are at higher risk of developing CVD. We will test new drugs in these disease models to eliminate symptoms. This new information and knowledge will be published and made available to other scientists and the general public and have great potential to translate into better clinical care for patients with these conditions.

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

The data generated by this project is expected to yield numerous benefits in the short term. Various groups stand to gain from the knowledge acquired through these studies, particularly scientists and clinicians working in the fields of reproductive health and cardiovascular medicine. They will directly benefit from the insights gained, which will provide a better understanding of the underlying mechanisms behind pregnancy complications and cardiovascular disease.

In the long term, pharmaceutical companies will also benefit from the outputs. The identification of novel pathways could be targeted for developing diagnostic tools, and novel compounds for therapeutic purposes. We will test and validate compounds that are effective for these conditions. Ultimately, both patients and clinicians will benefit from the outputs. The development of novel treatment options for conditions such as preeclampsia and other cardiovascular diseases will lead to improved therapeutic interventions for these conditions.

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

Our research findings will be disseminated through various channels to ensure broad exposure. We intend to publish our discoveries in reputable scientific journals, encompassing both the fields of reproductive health and cardiovascular medicine.

Additionally, we will target multidisciplinary scientific journals to maximize the reach of our findings. In order to engage with the scientific community, we will present our results at relevant scientific conferences and gatherings, attended by experts and clinical researchers.

We will establish collaborations with scientists in the field and Pharma (drug) companies to help us to identify and screen novel compounds for the treatment of these conditions.

In collaboration with our clinical associates, we will maintain regular communication with the patient information panel. Their invaluable insights will guide the direction of our investigations. In the meantime, we will keep them informed by sharing our findings and knowledge with them. Additionally, we will organise community education and outreach events on a regular basis to disseminate our research outcomes to the general public.

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 mouse because (1) mouse is the lowest organism that models complex diseases with good translation to humans; (2) different mouse models of cardiovascular disorders such as preeclampsia and atherosclerosis have been established with good reproducibility; (3) mice are amenable to genetic manipulations and many gene knockouts have been generated in mice and ready for use.

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

Model generation: We will generate different cardiovascular disease models using three approaches in wild-type or genetically altered mice:

Dietary modification:
Atherosclerosis: Atherosclerosis is a condition where fats, cholesterol and other substances are built up (plaque lesion) in and on the artery walls resulting in narrowing of the arteries and blocking blood flow. We will use established and appropriate animal models of atherosclerosis to induce lesion development through dietary modification, typically high fat diet. Wild-type and genetically altered mice will undergo approximately four weeks of dietary manipulation to induce plaque lesion development in atherosclerosis-prone strains. A compound or genetic materials will be delivered to the mice to modulate the pathways crucial to the development of lesion. At the end of the four-to-eight-week period, blood samples and tissue will be collected for analysis. The animals will have ad libitum access to modified diets.

Preeclampsia and hypertensive pregnancy disorder: Studies reported that high intake of sugar and polyunsaturated fatty acids were associated with increased risk of preeclampsia, yet, the underlying molecular mechanism is not fully understood.

Wild-type and genetically altered mice will undergo manipulation four weeks before pregnancy and during pregnancy. A substance or genetic materials will be delivered to the mice to modulate the pathways crucial to the development of preeclampsia. At the end of gestation, cardiovascular functions will be measured, blood samples and tissue will be collected for analysis. The animals will have ad libitum access to modified diets.

Injection of molecules

Preeclampsia is a hypertensive disorder during pregnancy. Pregnant wild-type and genetically altered mice will be injected with a substance to induce preeclampsia-like symptoms including hypertension and fetal growth restriction at mid-gestation. A substance or genetic materials will be delivered to the mice to modulate the pathways crucial to the development of preeclampsia. Cardiovascular function and fetal growth will be monitored during pregnancy. At the end of gestation, cardiovascular functions will be measured, blood samples and tissue will be collected for analysis. In some animal, cardiovascular functions will be monitored up to 12 months to assess the long-term impact of preeclampsia on the cardiovascular system.

Surgical operation
Vascular growth: some wild-type and genetically altered animals will have small pieces of tissue scaffold implanted under the skin and the growth of blood vessels monitored by non-invasive imaging. The animals will be housed under normal conditions, and tissue samples will be harvested up to 21 days, with regular monitoring. In some wild-type and genetically altered animals, blood flow to the hind limb will be disrupted by tying up or removal of the femoral artery and blood vessel regrowth monitored up to 21 days. These models will facilitate the examination of important genes in involved in the process of vessel growth. Blood flow and vessel density will be monitored using laser Doppler and immunohistochemistry analysis.

Reduced Uterine Perfusion Pressure - RUPP model of preeclampsia: blood flow to the uterine of pregnant wild-type and genetically altered mice will be reduced by tying up the ovarian artery to induce preeclampsia symptoms at mid-gestation. A substance or genetic materials will be delivered to the mice to modulate the pathways crucial to the development of preeclampsia. At the end of gestation, cardiovascular functions will be measured, blood samples and tissue will be collected for analysis. In some animal, cardiovascular functions will be monitored up to 12 months to assess the long-term impact of preeclampsia on the cardiovascular system.

Blood pressure will be measured using non-invasive or invasive methods in some animals. We will use volume pressure recording (VPR) sensor that are placed over the animal's tail to measure blood pressure in conscious mice. Some animals may have blood pressure measurements involving inserting a catheter to carotid artery with a radio telemetry device attached, which is placed along the right flank under the skin. Blood pressure will be monitored over the period of experiments.

We will use various imaging techniques to monitor blood flow and heart functions.

Laser Doppler imaging will be used to quantitative evaluate tissue blood perfusion. High-resolution ultrasound scanning will be conducted in pregnant mice to assess blood flow in specific areas of the placenta. Ultrasound scanning will be performed on pregnant and non pregnant animals to evaluate uterine, umbilical, cardiac blood flow and cardiac function.

Blood samples may be collected from superficial vessels at regular intervals.

Additionally, 24-hour urine samples may be collected from treatment groups.

Finally, various treatments or therapies may be delivered to the mice to modulate the pathways crucial to the development of different cardiovascular diseases.

Treatments can be delivered through different routes, including subcutaneous, intravenous, intraperitoneal injections, inhalation, oral administration through food or

drinking water or by gavage, mini-pump implantation, subcutaneous pellet implantation, or topical application to the skin.

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

The animals used in the different disease models will exhibit key features of those conditions, such as hypertension, impaired cardiac function or limb ischemia. Apart from these clinical signs, we anticipate various degree of impacts for the animals depending on the approach used to generate the disease model.

In the dietary modification or injection induced disease models, we anticipate that the animals do not display any significant adverse effects or abnormal behaviour. While injections may cause momentary discomfort or pain, they will not have any lasting effects.

In surgery operation induced models, animals may experience various degree of discomfort. Our models involve a surgical cut at the lower abdomen for preeclampsia model, or an incision over the femoral artery for hindlimb ischemia model, followed by tying up the ovarian artery or femoral artery. In the hindlimb ischemia model, the animals may experience different degree of walking difficulties. Some may have reduced mobility, reduced weight bearing and tissue damage on the limb that has been surgically operated on. The animals will be closely monitored for their functional outcomes. Generally, the discomfort may last up to 21 days (duration of the experiment).

In the RUPP model, we do not anticipate any significant adverse effects or abnormal behaviour, including preterm or aborted deliveries. Some genetically altered animals may experience aborted deliveries. We will closely monitor the body weight, signs of aborted litters, and activity of animals. Typically, experiments involving pregnant mice will span the duration of their pregnancy, which is approximately 19 days.

Studies exploring the impact of preeclampsia on the cardiovascular system will be conducted for a period ranging from 1 to 12 months post-delivery. We do not anticipate any significant adverse effects or abnormal behaviour these animals.

Animals undergone surgical procedures may experience discomfort that will last for a 24-48 hours and will be managed with painkillers.

The administration of treatments will primarily be through intravenous or intraperitoneal injections, and we do not foresee any adverse effects resulting from this dosing strategy. Our surgical procedures involve the insertion of a device under the skin to deliver substances (such as drugs) and will be performed under general anaesthesia with analgesia. We expect the animals to resume normal behaviour within a day of this procedure, although they may experience mild pain or discomfort, which will be managed with painkillers.

When measuring blood pressure in mice, it will be necessary to restrain the animals temporarily. This may cause brief discomfort, but it is unlikely to cause any lasting harm or stress. More invasive measurements of blood pressure involve insertion of a catheter to carotid artery attached to a radio telemetry device which is placed along the right flank under the skin. The procedure is taken under general anaesthesia. The animals generally recover quickly, with normal food and water intake within 24 h of surgery. On average, the animals return to presurgical body weight (subtracting the weight of the implant) by 4 days. Typically, experiments involving pregnant mice will span the duration of their pregnancy,

which is approximately 19 days. Studies exploring the impact of angiogenic genes on the cardiovascular system will be conducted for a period ranging from 4 to 12 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)?

The expected severity is mild or moderate. The majority of the animals involved in this project will experience a level of discomfort no greater than mild severity (70%).

These discomforts will be caused by interventions and procedures that only result in temporary and mild discomfort, without causing any lasting harm. In cases where repeat procedures are necessary, such as measuring blood pressure while the animals are restrained or assessing blood flow using laser Doppler and ultrasound, or surgical operation is performed, the severity may increase to a moderate level (30%). Animals that undergo general anaesthesia, including instances where it is administered multiple times, may experience a recovery period that could also reach a moderate severity level.

What will happen to animals used in 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 perspective is that utilizing alternatives to animals cannot effectively achieve the aims and objectives of this study. Cardiovascular disorders are complex conditions, which may lead to multi- organ dysfunction. Although progress has been made in in vitro models to study aspects of disease processes, these in vitro models do not provide important pathophysiological understanding into disease mechanisms particularly on an organ and systemic level. For example, tissue culture systems introduce significant alterations to the characteristics of endothelial cells, lack the contextual relevance of extracellular matrix molecules and interstitial cell types, and fail to realistically replicate the time course of adaptations. In animal model, the integrative nature of tissue function in its original environment becomes crucial to address the problems at hand. Thus, the scientific rationale of this study is best served by utilizing animal models.

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

We are also striving to reproduce certain research studies using in vitro methods. If successful, this advancement could potentially reduce the need for animal experimentation in future research endeavours. We use primary human cells or human cell lines to investigate the cellular and molecular mechanisms of the conditions. We also use these cultured cells to test the efficacy of drugs. We will also use tissues from human patients after surgery on condition that ethical consent is approved.

Why were they not suitable?

While in vitro systems, such as cell cultures, offer valuable tools for analysing molecular pathways, they fall short when addressing the complex questions, we seek to answer at the level of the entire organism. To truly understand how different cell populations behave and interact within a complex environment, it is imperative to observe them within a living animal. This level of complexity cannot be adequately captured through cell culture-based experiments.

Nonetheless, we will fully utilize cell-based experiments where possible. This approach allows us to refine our experimental design and minimize the number of animals required. Additionally, we will incorporate appropriate human samples to facilitate the translation of our findings from mouse studies to human populations.

Moreover, in order to bridge the gap between fundamental biomedical research and practical applications or protocols in clinical settings, it is essential to validate key hypotheses using pre-clinical models that we will develop throughout the duration 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 determination of the required number of animals typically involves the utilization of a mathematical model. This model aids us in calculating the minimal number of animals necessary for our purposes.

Specifically, it enables us to conduct experiments aimed at detecting significant differences between groups of animals, while ensuring that any observed disparities are not merely due to random chance.

We may rely on previous experiences, either from our own work or documented in the literature, to guide our selection of sample sizes. Some the long-term experiment was designed as longitudinal studies using the same animal, which will reduce the number needed for the experiments.

To validate our estimations, we will also assess them in light of new experimental tools available at the National Centre for the Replacement and Reduction of Animals in Research (NC3R) associated with this project.

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

Reductions in the utilization of animals will be accomplished by employing ex-vivo techniques, involving the extraction of organs or cells from mice, as well as in vitro

methods, where cells are cultured in a controlled environment. Additionally, meticulous experimental design incorporating power analyses and optimised breeding strategies will be implemented to minimize the number of mice lacking the specific genetic alteration being studied.

To minimize the usage of animals, we will maximize the utilisation of individual mice by conducting numerous observations and measurements. Moreover, we will collect a wide range of tissues and organs during post-mortem, even if they are not immediately necessary for a particular study. These collected samples will be stored in a tissue bank, enabling future investigations without the need for additional animals. This approach significantly reduces the necessity for using more animals in subsequent studies.

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

Our team possesses extensive experience in conducting such experiments, allowing us to implement efficient breeding strategies. We prioritise minimizing the number of animals involved by maximizing observations and measurements on individual animals. Given that each litter typically consists of multiple pups, this approach becomes more feasible.

Furthermore, we judiciously collect appropriatetissues for later analysis, thereby enabling the generation of multiple datasets from a single pregnant animal. Additionally, we aim to facilitate collaboration by allowing other researchers to access and utilize tissue samples from animals utilized in this study, whenever possible.

To further reduce the reliance on animals, we typically conduct preliminary work using cell cultures to inform the design of in vivo experiments. Cellular models are also employed to supplement and complement the findings obtained through in vivo experimentation. The design of our experiments and subsequent data analysis and reporting adhere to the guidelines set forth by the NC3R's PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) and ARRIVE (Animal Research: Reporting of In Vivo Experiment) protocols, respectively.

For each experiment, we meticulously develop comprehensive Study Plans. These plans encompass a clear statement of objectives, a detailed description of the proposed experiments (including treatments, experiment size, and materials used), and an outline of the methods employed for analysing the obtained 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.

The chosen animal models and methods for this project have been carefully selected to gain a comprehensive understanding of underlying mechanisms of some of the

cardiovascular disorders. These models are well-established, and have been extensively used and improved upon in the field. By utilizing this model, we can investigate the underlying mechanisms of these conditions in a controlled and consistent manner.

One of the methods we employ is adenoviral mediated gene transfer, which offers numerous advantages in studying atherosclerosis, angiogenesis, and vascular remodelling related to pregnancy. This method is highly efficient and less invasive compared to other approaches. In the era of personalised medicine, adenoviral mediated gene transfer has proven to be a valuable tool for inducing diseases and studying their progression. Previously, other models relied on delivering large volumes of proteins via mini pumps, but this had limitations in terms of dosage regulation and effectiveness. However, through our experience over the past five years, we have refined the adenoviral mediated gene transfer technique, ensuring its effectiveness and reliability in our studies.

In models where surgical operation are perform, such as RUPP, we have successfully adapted the RUPP model from rats to mice. In our model, we ligate ovarian arteries above the ovaries without ligating the uterine arteries. Our adapted approach has resulted in no adverse outcomes, including preterm or aborted deliveries. We believe that by reducing the severity of this procedure from severe to moderate, we can still obtain valuable insights into the pathophysiology of pregnancy-based disorders.

In hindlimb ischemia model, we adopted the femoral artery transection model, a mild hind limb ischemia model that mainly induces arteriogenesis in the thigh without calf angiogenesis. Animals recover from the operation fast and do not exhibit severe functional outcomes.

Because the operation may cause short term pain, we will consult with local vet.

Appropriate preoperation and postoperation analgesic agents will be given.
Our previous experience with these models has enabled us to refine the procedures, ensuring reproducible outcomes within a short timeframe. This approach minimizes the number of animals required for experimentation while maximising the knowledge gained.

Through active engagement in these studies and continuous refinement of techniques, we aim to proactively advance the field and contribute to the primary literature. Our goal is to uncover new knowledge that may have veterinary applications initially and potentially lead to advancements in human experimentation in the future.

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

The disease models we use involve applying different procedures such as vessel ligation, insertion of device, ultrasound scanning. It would be difficult to do given the small body size during animal early immature life stage. Pregnancy disorder models involve animals that have reached sexual maturity (between 6-8 weeks) which would mean animals at early life stage are not suitable for the study. Less sentient species, such as invertebrates, do not share similarity in anatomical or genetic basis and physiological functions with humans. Other less sentient species, such as zebrafish, have been increasingly recognised as useful animal models to understanding vascular development and human diseases. However, due to the dissimilarity of some organs it is difficult to use zebrafish as a model for respiration or reproduction in humans. Furthermore, the structural differences of the heart between zebrafish and human and the regenerative competence of zebrafish has limited the effectiveness of these models in the understanding of certain cardiovascular disorders, such as septal development and heart failure. In addition, water-

insoluble drug administration in zebrafish is another limitation.

Mice, on the other hand, share about 98% of DNA with humans, and they are biologically very similar to humans and get many of the same diseases, for the same genetic reasons. In addition, they can be easily genetically manipulated allowing for a deeper exploration of the molecular foundations of diseases.

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

We will implement various measures to improve the quality of life for animals based on the procedures performed.

For the non-invasive procedures such as diet modification, and time mating, we will closely monitor the general health conditions of the mice such as body weight, eating, drinking, movement, and social behaviours. In the event of an animals displaying abnormal behaviour such as failure to move freely, feed, groom or socialise normally the affected animals will be closely observed and the NVS or NACWO will be consulted.

We also provide "behavioural" training to mice undergoing specific procedures, such as tail cuff blood pressure measurements, to acclimatise the animals to the procedure. This training helps the animals adapt to the procedures more easily and reduces potential stress.

Non-invasive procedures involving anaesthesia, such as laser Doppler or Ultrasound scanning, animals will be put in a warm cage after procedures and closely monitored during recovery from anaesthesia and daily thereafter.

For surgical procedures, such as RUPP and hindlimb ischemia models, we have adopted approaches to ensure a quick recovery and prevent adverse effects. For examples, we have adapted the RUPP model which does not lead to any significant adverse effects or abnormal behaviour, including preterm or aborted deliveries. In hindlimb ischemia model, we adopted a mild hind limb ischemia model.

Animals recover from the operation fast and do not exhibit severe functional outcomes. Because the operations may cause short term pain, we will consult with local vet. Appropriate preoperation and postoperation analgesic agents will be given.

To monitor the health of animals during and after procedures, we utilize scoring sheets. These sheets allow us to track any changes or issues that may arise, enabling us to address them promptly and ensure the animals' well-being. Lastly, for animals that are likely to experience weight loss, such as those beginning a treatment where weight loss is expected, we offer mash with improved nutritional composition and palatability. This specialized diet is maintained throughout the entire duration of the procedure, helping to prevent excessive weight loss, and supporting the animals' overall health.

Repeated administration of treatments through intraperitoneal injections and gavage involves restraint of the animals which may cause stress. To reduce the stress, we will perform the procedures no more than once a day. Where possible minipump delivery system or administration via drinking water or food will be considered.

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

We will plan experiments and perform the procedures in compliance with the PREPARE, LASA and ARRIVE guidelines. Students, post-docs and academics who work under this licence will meet regularly to discuss issues encountered and refine the procedures accordingly.
Laboratory Animal Science Association (LASA) guiding principles documents of aseptic technique (https://www.lasa.co.uk/wp-content/uploads/2018/05/Aseptic-Surgery.pdf)
ARRIVE (Animal Research: Reporting of In Vivo Experiment) guidelines for preparing papers for publication (https://www.nc3rs.org.uk/arrive-guidelines)
PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for planning our experiments (15 topics including formulation of the study, dialogue between scientists and the animal facility, and methods) (https://www.ncbi.nlm.nih.gov/pubmed/28771074).

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

To ensure we stay informed about the latest advancements and opportunities for implementing new alternatives and refinements, we will refer to the NC3R's website, including the NC3R resource library, NC3R gateway, and Education and Training Resources in In-vivo Sciences. I'm also a member of the AWERB committee. This means I am in a good position to keep up to date on ethics and latest news from ASRU and NC3Rs. One noteworthy commitment we have made is in response to the recent initiative by the NC3R's to refrain from lifting mice by the base of their tail, even for brief periods, as this has been shown to increase their levels of anxiety and stress. Furthermore, we will stay updated on relevant literature in our field, actively participate in research conferences, and continually refine our experiments. Our goal is to reduce or replace the use of animals whenever possible. As new alternative models or approaches emerge that offer improvements over current practices, we will conduct studies, including pilot studies, to verify their effectiveness. We will ensure that all users receive proper training in adopting these new approaches.

59. Probing interactions between Tumour and the Immune System

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, Tumour, interaction based transcriptomics, Labelling tumour-Immune interactions, 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 primary objective of this project is to understand how the immune system interacts with cancer cells, with the aim of finding ways to enhance the immune system's ability to destroy tumours and to minimise factors that help tumours grow. This could potentially lead to the development of better cancer treatments and improved outcomes for cancer 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?

Cancer remains one of the leading causes of death worldwide, placing a significant burden

on healthcare systems like the NHS in the UK. While advancements in treatment, such as surgery, chemotherapy, and radiotherapy, have improved survival rates, these approaches often have limitations, including severe side effects and the potential for relapse. Immunotherapy, which harnesses the body's immune system to fight cancer, has emerged as a promising alternative, offering significant breakthroughs in recent years. However, not all patients respond to immunotherapy, and the reasons for this variability are not well understood. This research is crucial because it seeks to address a critical knowledge gap: the intricate interactions between immune cells and cancer cells. Current treatments are often limited by a lack of comprehensive understanding of these interactions.

Immunologists may not fully grasp the complexities of tumor biology, while cancer biologists may lack in-depth knowledge of the immune system. This interdisciplinary project aims to bridge this gap by employing cutting-edge technologies to observe and analyze these interactions in real-time within living organisms. By identifying the specific genes and pathways that govern the interactions between immune and cancer cells, we hope to uncover new molecular targets that could lead to more effective therapies. The research could significantly advance our understanding of how to strengthen the immune system’s ability to combat cancer, potentially leading to improved patient outcomes and the development of novel therapies. Without this research, the potential for improving immunotherapy and discovering new therapeutic targets could be lost, leaving gaps in our ability to effectively treat cancer. The work proposed here could pave the way for the next generation of cancer treatments, making it an essential endeavor in the fight against this devastating disease.

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

The proposed research aims to generate new knowledge about how immune cells and tumour cells interact during the three stages of tumour growth: elimination (when the immune system detects and destroys cancer cells before they can form a tumour), equilibrium (when the immune system controls the cancer, preventing it from growing but not completely eliminating it), and escape (when cancer cells evade the immune system, grow, and form a tumour). By identifying the specific molecules and immune cells involved in these stages, we hope to understand how tumours eventually manage to escape the immune system. This research could uncover new targets for treatment, which might lead to future clinical trials, either as standalone therapies or in combination with existing ones. The findings will be shared through publications in leading scientific journals and presented at international conferences, adding valuable insights to the field of cancer immunotherapy.

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

Cancer immunology has witnessed remarkable transformations in recent decades, thanks to the breakthroughs in cancer immune therapies. However, a significant challenge in tumor immunology lies in the limited understanding of tumor biology among immunologists, while cancer biologists often lack profound knowledge in immunology. The proposed research aims to investigate the intricate cell-cell interactions between immune and tumor cells, which inherently requires an interdisciplinary approach. This research holds immense potential to benefit the fields of cancer biology and cancer immunology, benefiting scientists in both academia and industry. Furthermore, the exploration of tumor-immune interactions will significantly benefit research groups in academia and industry that are focused on studying therapeutics in tumor biology. Finally, my research will generate a substantial volume of quantitative data concerning tumor-immune interactions, which can be used as a resource for both cancer biologists and tumour immunologists.

- Specifically, outputs of this work will directly benefit:
- members of my research group, it will allow us to test our ideas and further advance our knowledge of tumour immunology
- other researchers within the fields of cancer biology, immunology, cell-cell interactions and computational biology
- clinicians who may translate the research findings into practical applications and potential therapeutic strategies
- biotech and pharmaceutical companies that are developing novel cancer immunotherapies
- in a long term cancer patients Short term benefits:
- Understanding on a single cell level which immune cells interact with tumor cells and how the dynamics of these interactions changes as tumours progress.
- Medium term benefits:
- Understanding molecular mechanisms that govern tumour immune interactions. Long term benefits:

Developing new therapeutic agents and regimens that target on the molecular level tumour-immune interactions.

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

To maximize the output and ensure the impact and significance of this work we will employ several strategies. Rigorous experimental design will be employed, ensuring careful planning to minimize experimental variability and increase the validity of the findings. The establishment has a unique community of immunologists, cancer biologists, technologists, and state-of-the-art facilities and resources, which will enhance the quality of the research. Furthermore, the institute boasts a group of highly talented computational biologists who will employ advanced data analysis techniques to extract meaningful insights from the vast datasets generated by the cell-cell interaction tracing technologies. Effective analysis will uncover valuable patterns and correlations. I will aim to publish the research findings in top-tier scientific journals, reaching a broader audience and increasing the visibility and impact of the work. Additionally, I will present the research findings at conferences and seminars, engaging with the scientific community and welcoming feedback. To promote transparency and collaboration, I will ensure that after publication, my research methods and data are made accessible to the scientific community. This will allow for reproducibility and further collaborative efforts. In relevant cases, I will collaborate with clinicians to translate the research findings into practical applications and potential therapeutic strategies. Moreover, I will consider the long-term implications of the research and plan for follow-up studies to validate and build upon the initial findings. By adopting these strategies, I intend to make a valuable contribution to the cancer immunology field.

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.

The immune system consists of various immune populations examples include: dendritic cells, monocytes, macrophages, neutrophils, T cells and B cells the majority of which cannot be differentiated in vitro even as a single population. In vivo during tumour growth all these immune populations are present within the tumours at different stages of differentiation and this system cannot be mimicked in vitro.

Additionally, tumour cells grow in vivo differently than in vitro and also undergo selection mediated by the host immune system and other biological factors a lot of which are currently unknown and therefore also cannot be substituted for the in vitro system. Since we are planning to test different therapeutic agents, we cannot conduct these studies in humans. In addition, all the technologies that we use to probe cell-cell interactions are based on unique genetically altered mouse lines.

Explicitly all the mice express bacterial enzyme sortase, which allows to trace interactions between different cells in vivo. Our previous studies showed that cell-cell interaction undergo different dynamics in vitro vs in vivo and therefore we cannot employ in vitro systems. Experiments will be conducted on adult mice, in order to recapitulate the majority of natural cancer development as in patients.

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

Tumor cell lines, such as those from lung cancer, liver cancer, pancreatic cancer and metastatic melanoma, will be introduced into young adult animals via different routes. These methods allow the cells to enter the tumour target organ e.g lung tissue, resulting in the development of tumors or tumor metastasis. Alternatively, various transplantable tumor cell lines may be injected subcutaneously into the flanks of mice (one cell line per injection). In genetically engineered mice, tumors will be induced by administering agents that activate oncogenes, for example tamoxifen in the case of pancreatic tumours or viral delivery for lung cancer. The tumors will be allowed to develop for a minimum of several days and up to 4-5 weeks, or even longer periods of 2-3 months for genetically induced tumors or when assessing animal survival post-treatment. During this period, the animals may receive various drugs administered orally by gavage or through injections, with frequency varying according to the specific treatment regimen. For subcutaneous tumors, their size will be measured by calipers every other day starting from 7 days post-injection. In the case of lung tumors, pancreatic tumours, or liver tumours size assessment may be conducted using in vivo imaging techniques, such as micro-CT for lung cancer and ultrasound or MRI for liver and pancreatic cancers. Mice will be euthanized at different time intervals, ranging from 48 hours to 2-3 months post tumor injection.

Approximately two hours prior to euthanasia, a substrate will be administered either subcutaneously or intraperitoneally to trace cell-cell interactions. Subsequently, tumor tissues and tumor-draining lymph nodes will be collected for analysis. In all experiments, we aim to minimize the duration necessary to answer the scientific question at hand and to employ the least invasive methods possible.

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

In our research, we focus on tumour development in the lung, liver, pancreas or subcutaneously, tumour-immune interactions that happen during tumour development and potential therapies that may influence tumour-immune interactions.

We meticulously monitor the welfare of the mice to minimise animal discomfort. If tumours in subcutaneous locations grow, we have strict controls in place regarding to tumour size and to the amount of ulceration that tumors may exhibit. For example, if there is an open ulcer, the animal is immediately euthanized.

When it comes to pulmonary tumours an increase in size could hinder breathing. We preemptively address this by humanely killing any mice that suffer from respiratory distress. Additionally the assessment of pulmonary tumour size via micro-CT scanning aids in identifying large tumours and prior to tumour burden becoming excessive. Based on the CT data mice with large lung tumours will be humanely euthanized. For pancreatic and liver tumours animals may display various phenotypes related to the metabolic function of these organs. Animals will be meticulously monitored for displaying any signs of diseases. In addition, therapeutic drug treatments may cause adverse effects. We counter these risks by careful observation of the mice's health, opting to humanely kill those that demonstrate loss of weight or deteriorating health conditions. Importantly, the presence of strong clinical symptoms is minimised, as these are definitive endpoints that determine the completion of our 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)?

Around 45% of animals on this project will reach mild severity as they will be used in breeding and maintenance of genetically altered mouse strains or as control animals with no procedures or minimal e.g. injection of labelling agent.

The reminder of animals 55% of all animals on this project, will be used as cancer models and have a potential to reach moderate severity either from tumour burden or combination of the procedures.

What will happen to animals used in 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 study of human cancer advances greatly from the examination of actual tumour specimens and cell cultures. Yet, for a thorough mechanistic insight into tumour-immune system relations, live animal models are indispensable. As previously mentioned, the dynamics of tumour progression and the shifting landscape of the immune response cannot be effectively mimicked *in vitro*. My work makes extensive use of innovative technologies that facilitate the study of cellular interactions within live organisms. These state-of-the-art methods employ a variety of genetically altered mouse strains, specifically engineered to express the bacterial enzyme sortase — a currently exclusive option for

such *in vivo* research. This kind of work exploring *in vivo*, a single cell level resolution of interactions between immune system and tumour cells is novel and not yet published. Our in-depth studies performed outside a living system have demonstrated that the detailed dynamics of cellular interactions inherent to living organisms are not replicable *in vitro*, hence the essential use of live mouse models. Furthermore, to corroborate hypotheses that stem from our expanded knowledge of the dynamics between tumours and immune responses, the use of mouse tumour models is essential. These mouse models employing sortases to trace cell to cell interactions between immune system and tumour cells *in vivo* have never been previously done and will provide novel insights regarding dynamic communication between immune system and tumour that cannot be mimicked *in vitro*. Once we gain some understanding of the tumour-immune interactions *in vivo* some replacement alternatives will be employed (see next section). In summary, mice are a well-established and documented model for human cancers, with cell-to-cell interactions and immune functions that closely resemble those in humans. The relevant GAAs are either readily available or can be generated with relative ease, making them suitable for the proposed research

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

We have tried tumour cell lines and human tissue, but without tracing cell-cell interactions, since our studies in mice show drastic differences between interaction kinetics *in vitro* and *in vivo*, which basically implies that at the moment physiological tumour-immune interactions can only be studied *in vivo*. The dynamics of interactions in living systems are too complex and unfortunately cannot be replaced by computer modelling or *in vitro* systems.

We can't fully recreate how tumours and the immune system interact in a lab dish.

However, we can study some parts of this interaction between specific immune cells and tumour cells *in vitro*. First, we will identify which main types of immune cells interact with tumour cells. Then, we'll try to recreate these interactions *in vitro* using immune cells from mice. If we have suitable cell lines for these immune cells, we'll use those instead to reduce the number of mice needed. Once we know which cells show antigens (antigen-presenting cells) interact with different T cell types, we'll culture these cells together *in vitro*. This will help us reduce the number of mice used, especially if we can use existing cell lines. We will also test how treatments affect immune and tumour cells *in vitro*. This will help us choose treatments that minimize the use of mice in experiments. Additionally, we'll generate a lot of single-cell data that can be applied to human studies. This data will help us understand how the immune system affects tumour growth, further reducing the need for mice. We have performed searches in the following sources for various alternatives to in vivo work proposed:

European Commission datasets on replacement of animals specifically in immuno-oncology research, breast cancer alternatives and on alternatives to cell therapies
3Rs resources on NC3Rs pages, NORECOPA database and on other publicly available 3Rs databases Publication search engines for alternatives/replacement
There is no technology available in vitro that allows us to look at the interaction between cells at the resolution provided by LIPSTIC system.

Why were they not suitable?

In vitro cell-cell interactions fail to fully replicate the *in vivo* behaviour of interacting cells. Additionally, the complexity of the evolution of tumour and immune system as tumours

develop is currently not possible to recapitulate *in vitro*. There are too many immune populations that change their phenotype and gene expression patterns constantly as tumours develop and it is not feasible to capture these changes *in vitro*, not to mention interactions between these constantly changing immune cells and tumour cells. Moreover, *in vivo* validation is essential to experimentally confirm various hypotheses derived from an improved understanding of tumour-immune interactions. Furthermore, the testing of various therapeutic modalities that aim to target tumour-immune interactions also necessitates the use of mouse tumour 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?

I have calculated the required minimum number of animals to ensure that our experiments yield statistically valid results. Over the past 12 years of my research into tumour immunology using mouse models, I have been able to ascertain the optimal sample sizes.

Each experiment is repeated two to three times to avoid random and systemic errors and ensure reproducibility of the observation, depending on the achievement of statistically significant results with a total number of approximately 8- 10 animals per experimental group for transplantable tumour cell lines. For inducible tumours there is much more heterogeneity and approximately 15 mice per group are needed. For example, if I want to test the effect of a new drug on tumour-immune interactions together with a standard of care treatment. I need a minimum of four groups with (10 transplantable + 15 inducible tumours) mice in each group = 100. If I want to test interactions at two stages of tumour growth early i.e. prevention, intermediate i.e. intervention or late i.e. regression. I will need approximately 200 animals. For 5 new drugs I will need 1000 mice. I will work with 4 cancers subcutaneous, lung, liver, pancreas. I will mostly focus on the lung and subcutaneous cancers and will use only one time point for liver and pancreatic 1000 x 2 + 500 x 2 = 3000. We also want to look at interactions between antigen presenting cells and T cells. Assuming exactly the same logic another 3000 mice approximately will be needed.

Finally looking at the interaction between 2 specific immune cell types and tumour cells (in this case looking at what happens to the tumour cells), assuming the same calculations 6000 mice will be needed.

Additionally, mice will be used for breeding (various LIPSTIC crosses to specific Cre lines etc). Consistent with ethical research practices, I utilized my previous experience working with those genetically altered strains to project the number of animals necessary for breeding. This approach is guided by the principle of reducing animal use while still obtaining valuable and impactful data.

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

Where relevant we will conduct small pilot studies to optimize experimental conditions and

ensure that the chosen model is suitable before proceeding to the main experiment. We will use power analysis to determine the minimum number of animals required to achieve statistically significant results, which will help avoid using more animals than necessary.

We will use online tools, like the NC3Rs Experimental Design Assistant, for designing experiments that reduce animal usage while maintaining scientific validity. Where possible we will try to refine experimental techniques to minimize variability.

This, hopefully, will reduce the need for additional animals. Where possible we will do sequential experiments analysing data as it is collected and if statistical significance is reached, we will spare additional animals. If other laboratories are doing similar experiments, we share animal tissues and this will also potentially reduce number of animals needed.

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

To optimize the number of animals used in the project, in addition to good experimental design, we will implement several measures. Firstly, we will collaborate with the Colony Management team in order to create efficient breeding strategies, select appropriate strains or genetically modified animals that better represent the research question, thereby reducing the overall number of animals required. Additionally, where necessary we will freeze mouse embryos so that required genotype can be immediately rederived without excessive unnecessary breeding.

Also, we will only use inbred strains of mice to maximize reproducibility and thereby minimize number of mice needed. Additionally, we will conduct pilot studies to assess the feasibility of the experimental setup and identify any potential issues or improvements so that in actual experiments minimal number of mice is used. Where feasible we will strive to collect as much data from a single animal as possible. For example, for each experiment involving lung tumours we will use micro-CT to determine tumour size, we will also use tissue samples for histology and for qPCR as well as for flow cytometry. This will ensure that excessive numbers of animals is not used and unnecessary repetitions are avoided.

We will also make use of relevant literature and online tools, such as gene interaction networks and pathway analysis, to design the most relevant experiments and avoid unnecessary usage of additional animals. Where feasible, we will utilize in vitro studies, such as cell cultures or tissue-based assays, to complement in vivo data and reduce the need for live animals.

Collaboration with other research groups inside and outside the research institute will enable us to share samples and data and promote the efficient use of available resources, further reducing animal usage. Moreover, the institute strictly adheres to ethical principles and regulatory requirements. We will regularly review our protocols to ensure that animal use is justified, and whenever possible, we will consider alternative methods to minimize animal involvement.

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 research, distinct varieties of mouse models are employed to study lung cancer or melanoma metastasizing to the lungs (cell lines) as well as various subcutaneous tumour models of cancer (melanoma, colorectal, sarcoma, pancreatic and other cell lines available at the institute), liver cancer and pancreatic cancers. Both genetically modified models and models based on cancer transplantation will be employed. We will also use genetic model of pancreatic cancer induced by tamoxifen administration. The genetically modified models are not subjected to procedures before tumours are initiated through gene-altering substances (viruses or tamoxifen). Tumour formation is localised to the lungs by intratracheal delivery of these substances, mitigating the risk of system-wide effects. For pancreatic cancers tumour location is localized to the pancreas due to the specific expression of cancer driving genes exclusively in the pancreas. Similarly, intra-venous tumour injections confine tumour development to the lungs. For subcutaneous tumours, a maximum of two subcutaneous locations will be used, also minimizing overall harm to animals.

We have established strict controls on tumour dimensions to prevent distress in the mice and strict ulceration controls in case of subcutaneous tumour transplantations. For liver cancers tumours will be injected using ultrasound guided injection method via portal vein or spleen or directly into the liver. We adhere to stringent health monitoring, including weight monitoring, to ensure the mice are euthanised before strong signs of distress or poor health manifest. The selection of tumour models for this study parallels ongoing clinical and laboratory-based research, aiming to enhance both. The procedures detailed in the protocols are selected to balance the minimisation of animal use and discomfort with the need to obtain solid scientific data. For most orthotopic transplantation procedures, which introduce tumour cells into the lungs or liver to promote tumour growth at the site of origin, we utilise tail vein injections (lung) or portal vein (liver). All invasive recovery operations such as injection of tumour cells into the portal vein to model liver cancer are minimised and carried out only as necessary; pain relief and anaesthesia are administered as needed.

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

The choice of using mice for studying the immune system's role in controlling tumour growth is grounded in several key factors that align with both scientific principles and ethical considerations. Mice possess a biological and genetic similarity to humans that is essential for comprehending complex biological processes. This similarity is much closer when compared to other classical model organisms such as worms, flies, and zebrafish. Mice immune systems exhibit relevant parallels, making them particularly well-suited for investigating the intricate interplay between the immune response and tumour development, which is again much less the case for other classical model organisms. We chose to initiate tumours in young adult mice, because it most closely recapitulates initiation of tumour growth in human patients and therefore, we could not initiate tumour growth in even younger mice. Terminally anesthetized animals could pose limitations in understanding dynamic immune-tumour interactions. The ability to observe real-time responses and interactions in living organisms is crucial for deciphering the nuances of immune control over tumour growth. Anesthetized animals may not accurately reflect

these dynamics. In summary, while considering less sentient animals or those at different life stages is a thoughtful approach, the choice of the mouse model is rooted in the need to achieve scientific rigor and relevance in studying the complex role of adaptive immunity in tumour control, most closely resembling cancer patients. The ethical use of the mouse model, supported by its biological proximity to humans, enables us to attain valuable insights that could potentially translate to advancements in human cancer treatments.

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

Refining procedures to minimize welfare costs and harms for animals is a crucial aspect of ethical research. Our project is committed to employing a comprehensive approach that prioritizes the well- being of the animals involved. We will implement the following strategies to ensure the highest standards of refinement: Pre-Study Planning: Thorough pre-study planning will allow us to anticipate potential sources of distress or discomfort for the animals and to mitigate these factors where possible.

Pilot Studies: Conducting pilot studies will help us assess the potential welfare costs of the procedures. This allows us to make necessary adjustments before the main study, minimizing any negative effects on the animals. Tumour models were chosen to minimize the overall tumour burden in the mice, while closely mimicking lung, liver and pancreatic cancers in humans. Monitoring and Observation: animals bearing tumours will be monitored frequently to make sure that minimal harm is done to the animals. In cases of internal tumour models imaging studies will be conducted to make sure that tumour sizes are in check. If animals undergo surgeries, pain relief measures, such as analgesics, will be administered as needed to alleviate discomfort. Veterinary Care: Collaborating closely with veterinary professionals will ensure that animals receive prompt and appropriate medical attention whenever necessary. In addition, veterinary team has a very clear criteria by which they make a decision to sacrifice animals if they see signs of health deterioration. Alternative Techniques: Exploring alternative techniques such as *in vitro* studies and computer modelling where possible will ensure that minimal number of animals is used.

Regularly reviewing our procedures and protocols in light of new research and advancements will ensure that we remain up-to-date with the latest techniques for minimizing harm.

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

In conducting experiments with a strong emphasis on refinement and animal welfare within UK, our research project will adhere to best practice guidance established by reputable organizations and regulatory bodies. National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs): This UK-based organization offers extensive guidance and resources to promote the 3Rs principles.

Their guidelines cover a wide range of topics, including experimental design, pain management, and refinement techniques. FELASA Guidelines: While based in Europe, the Federation of European Laboratory Animal Science Associations (FELASA) provides valuable guidelines for laboratory animal care and use. Many of their recommendations are relevant and applicable within UK as well. Laboratory Animal Science Association (LASA): LASA is a UK organization that provides guidance on animal welfare and research practices. They offer publications and resources related to best practices in laboratory

animal science. Our research project will collaborate closely with the institution's ethical review boards and animal welfare and ethical review bodies. These local committees ensure that our procedures align with the best practices endorsed by the institutes and UK regulations.

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 Institute Observers: 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

LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. A report by the LASA Education, Training and Ethics section. (M. Jennings and M. Berdoy eds.) https://www.lasa.co.uk/wp- content/uploads/2018/05/Aseptic-Surgery.pdf
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

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

Staying informed about advances in the 3Rs (Replacement, Reduction, Refinement) and effectively implementing these advances throughout the project is vital to ensure the ethical treatment of animals and the quality of research outcomes. To achieve this, our approach involves several proactive strategies: Literature Review: Regularly reviewing scientific literature focused on animal welfare is essential. This will help us stay current with the latest advancements and best practices in the field.

Institutional Guidance: Collaborating closely with NIOs, will ensure that we stay informed in advances in the 3Rs. Our institution constantly receives updates about the 3Rs ensuring that all researchers stick to the most up to date standards.

I will make sure that all members of my laboratory will receive regular training and courses that address proper animal handling. Periodic reviews will be conducted in order for us to identify areas where the implementation of 3Rs advancements can be integrated effectively. Promoting open communication within the laboratory members will ensure that any new 3Rs advancements or ethical considerations are shared and discussed, leading to informed decision-making. We will adapt experimental protocols based on new findings and advances in the 3Rs. We will have discussion regarding 3R with other laboratories working with mice inside the institution.

60. Regulation of heart development in vertebrates

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

Heart, Embryos

Animal types	Life stages
Xenopus laevis	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 improve knowledge of early heart 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?

Cardiovascular disease is the biggest killer in developed world. An efficient way of treating heart failure will be to replace the lost heart tissue with new tissue, ideally from the same patient. Whilst this is not possible now, an emerging field of heart repair, or cardiac regenerative medicine, is enabled as the direct result of better knoweldge of heart development.

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

Improved knowledge of early heart development.

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

Any benefit is likely to be long-term. This project will contribute to large-scale efforts by workers in this field and may lead to improvements in cardiac regenerative medicine by providing better methods for making new heart tissue. Another potential benefit is better understanding of the mechanisms that cause congenital heart disease.

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

The outputs of this work will be published and presented at appropriate conferences. The work will further develop existing collaborations and open the new ones.

Species and numbers of animals expected to be used.

- Xenopus laevis: 100 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.

As it is difficult to study early heart development in mammalian embryos which develop internally, we are using frog embryos instead. Frog embryos are our choice model, as they are easy to obtain in large numbers, they develop rapidly under very simple culturing conditions, and also because they do not require heart for early development, so any experimental manipulation or mutations affecting the heart will be tolerated for several days. At the same time, frogs are vertebrates that share many features of development with mammals, including us, so most findings obtained from studies on frog embryos will be of broader significance.

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

We almost exclusively obtain embryos for our work from South African frog *Xenopus laevis* by natural matings. Egg-laying is induced by injecting human Chorionic Gonadotrophin (this was the basis for an early version of pregnancy test), a mild procedure that is repeated up to 4 times per year for each female animal.

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

A brief sensation of pain caused by needle is expected. Virtually all injected animals rapidly settle down. No adverse reactions were observed over the past 2 projects.

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 uses a single procedure, subcutanous injection of human Chorionic

Gonadotrophin dissolved in water. This is a mild procedure with animals often not showing any reaction to it.

What will happen to animals used in this project?

- Kept alive at the establishment for non-regulated purposes or possible reuse

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 focuses on early embryonic development of the heart and thus requires the use of animals. The frog Xenopus, along zebrafish is the lowest complexity vertebrate model used for studying embryonic development. At the same time, it is a vertebrate that shares many features of development with mammals and thus can partially replace their use. Most of the findings that we obtain from this non-mammalian model will be of broader significance.

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

Heart muscle cells can be made from human induced embryonic stem cells in culture, providing a powerful and popular in vitro model.

Why were they not suitable?

Even though the in vitro model is used in our group, there is still a requirement for a complementary in vivo model. Without an in vivo model, frog embryos in case of this project, it is difficult to evaluate the relevance of results obtained from an 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?

By using an estimated number of procedures per year (~100) and taking into account reuse.

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

The expansion of applications of human induced Plurpotence Stem (iPS; embryonic-like) cells for modelling human heart development and disease has led to a proportional reduction in use of animal models such as *Xenopus*. This shift has been enhanced by the corresponding changes of the funding climate. As a consequence, we are using *Xenopus*

embryos to complement our work with human iPS cells, instead of using them as an exclusive model for heart development as in our earlier work.

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

A form of reduction is our practice of using animals repeatedly to provide gametes after optimal recovery time. Embryos are obtained primarily by mating instead of in vitro fertilisation. Both practices act to greatly reduce the number of animals that would have been used 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.

We use embryos of the frog Xenopus laevis for our experiments.

We perform all experiments on embryonic forms before the onset of feeding. Adults are used to supply embryos using a mild procedure. Typically, no distress or lasting harm are observed.

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

As stated above, we are using one of the simplest vertebrate models, and our reserch focus is entirely on embryonic forms.

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

Animals recently used in procedures are monitored daily for several days. There is not much room for substantial refinement in a project which only uses a single mild procedure.

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

We follow well established practice in the field in which we have worked for 29 years. As we work with embryos before free feeding stage, refinement concerns only apply to adults used to produce embryos. The number of applications of a mild procedure per animal is limited.

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

I am regularly receiving NC3Rs news and I am continuously considering any which might

be relevant for our work.

61. Sarcomere Proteostasis in Titinopathies

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

Proteostasis, Titinopathy, Cardiomyopathy, Myopathy, Muscle

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 common DNA mutations in the giant muscle protein titin cause heart and skeletal muscle disorders.

To understand how the cell's protein turnover machinery is important in maintaining muscle health.

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?

Titinopathies are a collection of heart and muscle diseases caused by DNA variants in the titin gene (TTN). We will study DNA variants that cause the titin protein to become unstable (variants WR and PL), as well as a variant that causes titin protein to be shorted (truncating variant - frequently found in patients with the heart disease dilated cardiomyopathy). Understanding how these DNA variants affect muscle will help identify

disease-causing DNA variants and allow these DNA variants to be used for diagnosis and treatment.

It is thought that disruption to the cell's protein quality control machinery (proteostasis) is how these TTN DNA variants cause disease. In fact, disruption of proteostasis is commonly known to cause neurological conditions such as Alzheimers and Parkinson's, heart and muscle disease as well as multi-system conditions such as Vici Syndrome, which affects the brain, immune system and muscle. We will study the Q331R variant in the gene EPG5, responsible for causing Vici syndrome. It is important to find out as much as possible about proteostasis so we can try to limit the damage this disruption causes and thus improve symptoms for patients.

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

We will gain new information about how disease-linked DNA variants in the titin gene affect the production and behaviour of the titin protein and how they cause disease in muscles.

We will also gain novel information on how a variant in the EPG5 gene, responsible for Vici-syndrome, affects proteostasis and hence damages organ systems (brain, muscle and heart).

This information will be disseminated in the form of high impact research publications, PhD theses as well as being presented in talks and conferences.

Once we have validated the mouse models of these diseases we also hope to test candidate drugs found in our screen against mutated protein.

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

In the short term <1yr, we will complete work from previous PPL, resulting in publication of Vici syndrome model and titinopathy models. Researchers and the wider scientific community will benefit from publications and presentation of the characterisation of these disease models.

In the medium term (1-5 years), we will have new knowledge on how mutated titin protein affects muscle and how disruption of proteostasis damages muscle in general. Publication and presentation of these new knowledge will benefit the wider research community, helping to further research into proteostasis and disease and help advance therapeutic ideas in the pharmaceutical industry.

Researchers will benefit from being able to test drugs found in in vitro screens on cells from the animal models, which will benefit the pharmaceutical industry.

In the long term (after the project has ended), the information on the TTN variants can directly inform genetics screening services and diagnostic services on which TTN DNA variants, or combination of variants are likely to cause disease, thus directly benefitting patients and their families. Any positive results from the the drug screens from cells may eventually be improved and tested in the animal models and could become clinically useful.

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

We will aim to publish in open-access journals and share our results in informal retreats

and more general genomics conferences.

For the mice modelling the multi-system disorder Vici syndrome, we will provide tissues to colleagues who have expertise on the analysis of the nervous system. In addition , we will provide collaborators with samples so they can look at the ultra structure of the muscles containing mutant titin using cryo- electron microscopy.

Any high throughput data will be made available to researchers with similar interests.

Further examples include publication in open-access journals, presentation at scientific meetings, making resources such as data available to other researchers.

We will store and share tissues with researchers who have similar interests.

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 will use mice for our studies as we can alter or remove the same genes as in humans and assess what effect this has on their muscle size, structure and function.

We will mainly use adults to breed mice carrying the same combination of disease causing variants as in the patients and study their offspring at all stages. We will study the embryos from these mice when the gene alteration results in no live births. As the diseases are sometimes progressive with age, we will study young, adult and aged mice so we can study the progression of the diseases caused by these gene alterations.

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

Mostly, we will be using healthy mice for breeding to generate mice bearing a combination of DNA variants that are linked to the human diseases and their muscle structure and function studied post mortem. These mice will be kept in controlled environments (Protocol 1). Some mice will have allowed to develop clinical signs of heart and muscle disease before studying their heart and muscle post mortem (Protocol 2). We will cull and harvest tissue from mice at all life stages (embryo, young, adult, aged). (Protocol 1, 2 and 5). Some mice will be subjected to non-invasive behavioural tests to analyse muscle function (balance tests, grip strength analysis, Protocol 3).

Some mice will be sedated using inhalation anaesthesia for cardiac echo-cardiography to study heart function (Protocol 4).

Some mice will be treated with drugs (orally, injection or undergo a minor surgical procedure to introduce an implant). Implantation involves under anaesthesia, making a small cut in the skin at the back of the neck and making small 2 cm pocket under the skin and placement of a minipump within the pocket. The cut is closed with surgical clips and

pain relief will be given by injection before the mice awake. This will take about 5 minutes in total and the mice are expected to experience a mild to moderate level of severity. (Protocol 3,4 and 5).

Some mice will undergo food withdrawal, i.e. be put on a water only diet for up to 24 hours to induce autophagy. (Protocol 3, 4 and 5).

Some mice will undergo minor surgery to remove a portion of their sciatic nerve. Under anaesthesia, small cut will be made in the skin, and a small 1cm window will be made in the left thigh muscle, then the sciatic nerve exposed and a 1cm portion removed. The wound will be closed with surgical clips. This will take less than 5 minutes and pain relief will be given by injection before the mice awake. The mice will then be kept for 2 weeks to allow 30-40% muscle loss in the lower hind limb, before being killed. These mice are expected to experience a moderate level of severity. (Protocol 6)

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

Some animals with certain genetic alterations will experience cardiac or muscular symptoms, such as panting and shaking, weight loss or poor grooming due to muscle weakness. Any animals experiencing panting, shaking or weight loss of over 20% over 3 days will be culled immediately.

Some animals will undergo minor surgical procedures (implantation or denervation) but they are expected to recover quickly (within an hour after surgery) and will be given painkillers and post- operative care.

Ageing of mice over 15mths may result in age-related conditions such as skin infections, tumours, cataracts, arthritis and hair loss.

Removal of the sciatic nerve renders them unable to utilise their left hindlimb for no more than 2 weeks, but normal movement is not affected.

Some animals may have some weight loss (not more than 20%) due to drug effects or food withdrawal for 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)?

The majority of mice we use have no obvious defects and experience mild levels of severity. Sub-threshold: 80%

Mild: 15%
Moderate: 5%

What will happen to animals used in 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?

Muscles are complex structures, made of different complex cell types and fibre types to give different physical properties and they mature in a 3D environment where external mechanical cues are needed for maturation and alignment of their sarcomeres - this cannot be easily replicated in non-animal models.

A multi-system disorder like Vici syndrome cannot be modelled without the use of whole animals.

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

We studied induced pluripotent stem cells (iPSCs) containing the disease causing variants.

We studied the effect of titin disease linked DNA variants by overexpression of variant containing titin proteins in cells and looking to see how localisation or behaviour of the protein is affected.

Why were they not suitable?

The differentiated iPSCs are an immature version of heart cells and doesn't allow us to reliably phenotype the variant containing cells. They are also rather inhomogenously differentiated into heart cells so it is difficult to do quantitative biochemistry.

Overexpression of variant containing proteins in cells gives us limited information on the behaviour of the mutant protein as the overexpressed protein does not incorporate correctly into the sarcomere.

Furthermore, the overexpressed protein is present at an unphysiologically high level which tends to overwhelm the cell's normal homeostasis leading to incorrect localisation and frequent aggregation.

It is also impossible to study the effect of the mutations on the giant muscle protein titin in its entirety as it is too large to express in 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?

I looked back on previous year's usage for breeding and maintenance and also estimated numbers needed for experiments involving procedures based on published data.
What steps did you take during the experimental design phase to reduce the number

of animals being used in this project?

I analysed previously published studies and will carry out pilot studies as well as use online tools (such as the NC3R's EDA) to ensure a high likelihood of detecting a biomedically-relevant effect size with minimal 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 freeze sperm from the different mouse lines, thus allowing us to reduce our breeding to a lower number, knowing that we have backup.

We will use big data analyses (proteomics and RNAseq) to guide our experiments, thus reducing the amount of samples needed for low throughput biochemistry.

Using mice to generate models of inherited human conditions will allow us to assess the effect of the loss, or mutation of a single protein in a whole animal model. This allows us to analyse, for example, both cardiac and different types of skeletal muscle in a single animal, and thus reduces the total number of animals used. We will also share tissue with researchers studying muscle and proteostasis, as well as brain and the nervous system for EPG5 mice, thus reducing the number of animals used per researcher.

We will use efficient breeding strategies to ensure we generate a minimum number of animals to get the right types of animals (e.g. genotypes/age) we need.

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 used as they can be readily genetically modified to produce animals with disease-associated variants as they have similar muscle structure and genetics as humans. We will monitor the animals that are expected to display symptoms of human diseases closely and humanely kill any animals that begin to show any distressful symptoms to reduce potential suffering.

Our exercise model is non-invasive as it involves placement of a running wheel into the home cage, similar to what most pet rodents have in their cages.

Our behavioural tests are minimally invasive and leave no pain, suffering, distress or lasting harm apart from that which comes from normal handling of the mice.

Cardiac phenotyping by echocardiography allows us to measure heart function in mice repeatedly which reduces the number of mice needed.
Our model of muscle atrophy is the most reliable and least invasive method as it involves a

minimally invasive and quick surgical procedure to remove a portion of nerve. Using this model does not severely affect the animals mobility.

We will use drugs to induce cardiac symptoms but will choose the combination and doses which will produce the outcome we want but have the least detrimental side effects.

We will use food withdrawal to induce autophagy but will chose the shortest period needed to produce the desired effects in the organs of interest.

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

Similar studies on titin variants are ongoing in zebrafish, however zebrafish have 2 TTN genes, which makes it difficult to rule out compensatory effects from the second copy of TTN. Futhermore, there is limited ability to phenotype the symptoms of the disease caused by these variants in zebrafish, for instance, it is difficult to test exercise capacity, cardiac function or study different muscle types to see which are affected by the disease. In addition, zebrafish are developmentally very different to mammals as they can survive the early embryonic stages due to diffusion being sufficient until a much later stage.

Most of the animals studied will be killed by a schedule 1 method and their muscle analysed post- mortem. Only a limited number of animals will undergo procedures such as surgery and anaesthesia, most will undergo non-invasive tests such as grip strength analysis or voluntary running on an exercise wheel similar to those commonly found in cages of pet rodents.

We will study some variants at embryonic stages, however the muscle at this stage is immature and does not have a highly ordered structure compared adult muscle.

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

All surgical procedures will be carried out using anaesthetic and pain relief appropriate to the age and weight of the animals and will be closely monitored during post surgical recovery and their wounds checked for proper healing.

We often only see differences when the muscles of the mice are tested by increased exercise or muscle wasting. All procedures have been refined to use the most humane techniques possible to reduce distress to the mice (e.g. the replacement of forced treadmill running with voluntary wheel running, refinement of surgical techniques with minimal wound size).

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

We are constantly following published protocols, attending conferences, and make use of sites such as https://www.nc3rs.org.uk/ as well as informal discussions with colleagues. We will be guided by PREPARE guidelines and the ASPA code of practice.

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

We will attend NC3R training courses and keep up to date with their online material as well as stay informed of any changes from the home office guidance via frequent consultations

with the animal facility staff, NACWO, NVS and other colleagues in the field.

62. The role of extrusion and smooth muscle in asthma attacks and inflammation

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

Asthma, Inflammation, Gadolinium, Bronchoconstriction, Eepithelia

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?

We have identified a new etiology for asthma. We found that the inflammatory cycle stems from epithelial damage from excess crowding after an asthma attack. With this new knowledge we are aiming to find better therapies for patients, with a long term aim of transitioning this work into the clinic to benefit asthma 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?

Asthma is extremely common and on the increase. Most current asthma treatments stem from the idea that it is an inflammatory disease. Yet, the life-threatening feature of asthma is the attack or the constriction of airways, making breathing difficult.

The main approach is to manage symptoms, using albuterol to open airways and steroidal

anti-inflammatories to mitigate inflammation. While the current treatments have helped lessen asthma features, they do not prevent future attacks. We have recently published (Bagley et al., Science 2024) a novel in vivo model for asthma that instead focuses on the damage stemming from the mechanics of the asthma attacks. We find that bronchoconstriction within mice causes excess airway epithelial damage from a process we discovered called extrusion, which then leads to the inflammation and other features of asthma. Moreover, our current evidence suggest that this damage then leads to amplification of smooth muscle that causes further attacks. Indeed, asthma patients on all the current therapies have even more airway epithelial damage than what we triggered in mice, underscoring the importance for improved therapies that prevent this damage.

Importantly, we identified that an element used in a different formulation as a contrast dye in magnetic resonance imaging can block this damage, opening a new possibility to cure asthma. To test this model, we need to use a well-developed mouse model for asthma.

While mouse models have some limitations, it is the best model for asthma that does not use larger animals (sheep, pigs, etc.) where we can test numbers sufficient to warrant a clinical trial in humans, which we are currently investigating. Should we be successful, the relatively small numbers of mice we will need for this study will contribute to an entirely new approach that could finally treat asthma.

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

New etiology for asthma, hopefully resulting in publications and clinical trials.

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

Asthma affects a large percentage of the population with many fatalities. Our new model stands to offer a new therapy that could cure asthma, rather than just limiting its symptoms.

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

We have already initiated several collaborations with others studying asthma, lung fibrosis and lung cancer. I have and will continue to present this in not just publications but also in talks at meetings and seminars.

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 are using adult mice, as they have been well-developed for asthma studies, with several established protocols for immune-priming to cause asthma attacks and using post-mortem lung slices. We are using adult mice, as beginning immune-priming at this stage consistently produces an asthma response. Additionally, we will need adult mice for breeding purposes.

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

We will immune-prime mice consecutively using a variety of protocols by intranasally instilling house dust mite in saline for several weeks. Some mice will in addition to exposure to house dust mite will also be exposed to therapeutic agents, such as gadolinium or current steroidal anti-inflammatory therapies such as budesonide.

At this point, we will either sacrifice them for ex vivo tissue analysis or challenge them with increasing concentrations of inhaled methacholine, similar to what asthma patients experience during a diagnostic test. sacrificeDuring these challenges, we may add additional compounds that block extrusion or inflammation in either live mice or slices.

Additionally, we may restrict food or alter diet to a high protein diet intake during the priming period to test if intermittent fasting can help regenerate airway smooth muscle to a non-pathogenic configuration. Water consumption will not be altered.

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

The worst adverse effects are only moderate, when we challenge mice by causing an asthma attack. However, we have already done a dose response curve on anaesthetised mice to establish the lowest dose that creates the needed response without inflicting much discomfort. Any discomfort experienced during these experiments resolve within a day usually, and mice are observed at 24 hours after exposure to be displaying normal behaviour patterns indistinguishable from control 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)?

We expect ~30% of mice would experience a moderate response with ~70% having a mild response.

What will happen to animals used in 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 have already developed our studies in cell culture, which led to the compelling finding that excess cell density or crowding could damage an epithelial barrier.

However, since the airway architecture and downstream effects of asthma attacks cannot

be replicated in cultured cells, we needed to test in an animal model. Our results have demonstrated that the smooth muscle underlying the epithelia become altered in amount and orientation that is critical for causing an asthma attack. There is no way to model this within epithelia, as it requires many cells and forces to develop this architecture. Mice are the smallest animal that we can do our assays in, which are well-developed as an asthma model relevant to humans. Without them, we would not be able to monitor the inflammation that involves other cell systems than just the airway lining.

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

As we are interested in the airway epithelia we have used cell culture methods to experiment on monolayers of cell lines. These experiments have been used extensively to inform any potential animal work. We are currently also using cell culture models whenever possible such as to test how house dust mite and rhinovirus infections affect epithelial sheets alone. We have also used histologically stained human lung biopsies from asthmatic patients to confirm that the phenomenon of airway epithelial damage after an asthma attack is present even with current treatments.

Why were they not suitable?

Whilst cell culture methods have been useful for informing us of potential pathways to target in asthma. They do not represent real structures seen in real lungs. The important piece is that they lack the musculature that surrounds the airways and contracts to cause an asthma attack. Also, we cannot see how the damage from attack-driven extrusion affects the downstream symptoms, such as mucus secretion, inflammation, and airway remodelling. The complex interplay between multiple different cell types, extra cellular matrices and external mechanical forces cannot be modelled in a non animal model. This is the first time that this has been examined carefully so that even if we knew how to make complex models, we wouldn’t know what to model without analysing real asthmatic 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?

The numbers of animals required for this project have been considered in detail, based on our own experience, as well as on information from the literature. We have already developed a way to use post-mortem tissue for most of our experiments so that we will only need to use live studies on a minimal number of mice to confirm our findings from ex vivo lung slices.

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

As mentioned above, we use lung slices as often as possible, as this allows us to compare treatments within the same lung, as responses can vary between mice. This and using

lung slices allow us to use far few mice than if all challenges are live. For the live mice experiments, we used literature and anaesthetised mice to establish the highest methacholine dose that would yield results while causing the least harm to the mice. For comparisons between current treatments and gadolinium the NC3R's Experimental Design Assistant is used to minimise the number of mice required.

From refining our experiments, we find we are now able to get numerous types of data from a single mouse including but not limited to:

- Precision cut lung slices
- Measuring airway response by analyzing the change in airway area after inducing an asthma attack
- Measuring airway destruction by quantifying extrusions and denuding
- Measuring immune cells infiltrating the lung
- fluorescent imaging
- Analysing gene expression with and without treatment following an attack
- Protein analysis with and without treatment following an attack.

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 and already do share tissue, not just the lungs to others in the lab for pilot studies. We also go over study plans within our lab and with other asthma researchers to ensure that we are conducting the experiments the right way, so that we do not repeat the studies, due to poor experimental design. Effective breeding strategies are used after consultation with geneticists to minimise the number of 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.

Our lung slices have already been incredibly useful in honing the best dose and length of treatment needed before turning to live mice. We will continue to use this approach so that we can best refine the treatments we plan to use before we take them to live mice. We will be diligent during our live animal studies to continually monitor and refine our techniques to ensure least harmful and best practices are always performed. The use of intranasal instillation to immune prime mice, causes only short-term discomfort and is targeted to lungs reducing the discomfort and pain experienced by mice. Previous work using intraperitoneal injections to immune prime mice has a similar to more moderate phenotype in comparison, however the use of injections is more likely to induce systemic inflammation and can causes more pain to the mice when compared to use of intranasal instillation.

As mentioned previously, we extensively use precision cut lung slices from mice.This

technique reliably produces over 30 slices from just half the lobes from one mice. In addition, each slice has multiple measurable airways. This allows us to test multiple different treatments and compare within the same mouse, with no harm to the mouse, as this is conducted after death.

From refining our experiments, we find we are now able to get numerous types of data from a single mouse including but not limited to:

- Precision cut lung slices
- Measuring airway response by analyzing the change in airway area after inducing an asthma attack
- Measuring airway destruction by quantifying extrusions and denuding
- Measuring immune cells infiltrating the lung
- fluorescent imaging
- Analysing gene expression with and without treatment following an attack
- Protein analysis with and without treatment following an attack

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

The lungs have to be fully developed to be relevant for asthma, therefore, we use mice typically from 8 weeks old. We used to work on zebrafish but they don't have lungs, nor do fruit flies or yeast.

Terminally anaesthetised animals are not an appropriate model, as asthma development requires continuous exposure to an allergen to develop over several weeks.

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

The develop of asthma can have moderate harm to the animal, however we largely expect it to have only a mild welfare impact on the mice. With the potential of a moderate harm to the animal, we do monitor them daily to ensure they do not suffer unduly expect the next phase of this project to have even fewer harms, as the goal is moving to prevent airway remodeling that causes the attack with our inhibitors, as we are aiming to cure asthma altogether.

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

Along with doing literature searches on currently used protocols, we will do power calculations to ensure that the fewest mice are used for experiments. Our experiments are designed and conducting in accordance with the appropriate guidelines such as the ASPA code of practise and ARRIVE.

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

We stay informed through our networks with the nearby asthma researchers . We also read up on the 3Rs in workshops or from Biological service staff. We are also subscribe to the NC3R’s newsletter.

63. The role of genetic mutations in neurodevelopmental disorders

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

Neurodevelopmental disorders, Autism spectrum disorder, Neuron physiology, Neuron morphology, Development

Animal types	Life stages
Rats	juvenile, adult, neonate, pregnant, embryo
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?

Neurodevelopmental disorders (NDDs) are a group of conditions that emerge during childhood and significantly affect brain function. Examples of NDDs include intellectual disabilities (ID) and autism spectrum disorder (ASD), which result from impairments in the growth and development of the brain. This project aims to examine the role of genetic mutations in these impairments and determine whether common molecular pathways and pathologies link groups of different genes linked to NDDs.

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?

Intellectual disabilities (IDs) and autism spectrum disorders (ASDs) are co-occurring neurodevelopmental disorders (NDDs), that affect approximately 700,000 people in the United Kingdom (National Autistic Society, UK). This project aims to increase understanding of how neurons might differ in ID/ASD patients, potentially providing the tools for earlier diagnosis and better treatments.

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

This project will provide new information about the effects of genetic mutations in neurodevelopmental disorders, will advance knowledge in the field, and result in several publications.

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

The proposed research will provide new insights into how genetic mutations can lead to changes in brain cells and affect their development in neurodevelopmental disorders (NDDs), including autism spectrum disorder (ASD). In the short-term this project will lead to increased understanding as to the extent of which the large number of genes linked to NDDs 'converge' onto a much smaller number of key phenotypes and underlying molecular pathways. The key strength of the proposed research is that it allows the direct effects of genetic mutations to be more easily measured, in the absence of compensatory responses seen in other models typically used in this field of research. A major part of this research will involve testing the extent of reversibility of NDD phenotypes using the knowledge gained during the project. Therefore, by the end of the project we expect to have identified new drug targets that could be further tested by other academics and the pharmaceutical industry.

Approximately 1-2% of people in the UK are affected by NDDs and costs related to treatment, lost earnings and care and support for autistic people in the UK are estimated to be £32 billion per year. If key convergent phenotypes and/or molecular pathways can be identified, then in the medium to long term this could allow for the development of better therapeutic options and diagnostic tools for people with NDDs. Identifying strategies for reversing ASD/LD phenotypes will provide valuable insights into developing new ways to help improve the health of people with ASD/LD.

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

Results from the proposed work will be published in scientific journals, by communicating at international conferences, and by invited seminars. Information will also be provided on the applicant’s University website, and via social media. I will also seek to explore ways of engaging with autistic patients themselves, as well as with autism advocacy groups. Publications arising from this study will be published in line with the UKRI open access policy. Open access to the research prior to publication will be made possible by uploading to the bioRxiv preprint server.

Species and numbers of animals expected to be used.

- Rats: 3600
- Mice: 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.

This project will primarily use rats over mice. Relative to mice, rats have larger brains and exhibit a more complex group of testable behaviours, but still retain many characteristics that make them useful for lab research. Recent developments in genomic-editing technologies have facilitated the ability to manipulate the rat genome, thus spurring interest in the rat as a model for genetically linked disorders.

Several rat models of intellectual disability (ID) and autism spectrum disorder (ASD) are now available to use. These animals have already been screened for behavioural phenotypes prior to being made available for use by other labs. This project is focused on looking for common pathways affected in different models of ID/ASD, making the availability of a number of different models key to success.

Initial characterisations will be carried out in early adulthood (4-12 week old rats) to establish phenotypes in NDD models once development has ended. These animals will not be used for subsequent breeding of animals. Follow-up studies will explore the developmental profile of the key differences that we observe in NDD models when compared to control animals without the genetic mutation This will be achieved by recording at key developmental timepoints (eg from birth to two weeks of age) to determine whether these phenotypes are present at birth or appear during development.

This data will be important when designing studies to test whether there is a critical period during which therapeutic options might be most effective.

Some neurodevelopmental disorders (NDDs), such as schizophrenia, remain best characterised by mouse models. To allow for potential future studies in mice we will aim to do an initial characterisation of mice from the background strain (= do not have any genetic mutations and was used for establishing the genetic altered mouse models) in the brain areas we hope to study.

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

A subset of animals will undergo stereotaxic surgery, to allow for injections into the brain through small holes in the skull (known as craniotomies). This is classed as a moderate procedure that takes 1-2 hours, and animals recover well within a few days of the procedure. Analgesics are given before, during, and after the procedure to minimise pain. The surgeries are carried out aseptically.

A subset of animals will undergo cardiac perfusion under very deep anaesthesia from which animals do not recover, to best preserve tissue for imaging purposes. Cardiac perfusion is a commonly used technique whereby animals are deeply anaesthetised prior to injections being made into the heart to allow perfusion of solutions directly into the bloodstream. Typical perfusions will use artificial cerebrospinal fluid, phosphate buffered saline, or fixatives such as paraformaldehyde.
Animals will either be killed using schedule one methods (typically cervical dislocation), or decapitation following anaesthesia (to best preserve brainstem tissue for live cell

recordings).

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

Models of neurodevelopmental disorders (inc autism spectrum disorder and schizophrenia) typically show deficits relating to social behaviours, motor co-ordination and sensory hypersensitivity. Motor deficits, when observed, are subtle and have limited effects in relation to normal animal behaviour. Sensory hypersensitivity phenotypes can lead to issues with increased anxiety in certain animal models. For example, FMR1 knockout males display enlarged testes compared to controls. 70% of Syngap1 knockout animals display with low-level severity absence seizures (head-bobbing, occasional forelimb muscle contractions).

Animals will experience pain following stereotaxic surgery. This is mitigated by using peri-operative care plus analgesia before, during and post-surgery. Animals typically recover well and are eating and behaving normally within 24 hours. There may be issues with weight loss post-surgery, but this is not something I have typically observed in my previous 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)?

Rat: Moderate (20%), Mild (80%).
Mouse: Moderate (20%), Mild (80%).

What will happen to animals used in 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 and nervous system is an incredibly complicated structure. Understanding how it works is essential to appreciating how this can go wrong and what interventions might be effective in delivering relief. Animal models continue to be important tools for understanding disease mechanisms and for preclinical testing of potential therapeutics. To fully understand NDDs and achieve the aims of this project, intact animals need to develop normally in utero. This will generate testable hypotheses that can be tested in future experiments using non-animal alternatives.

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

We considered the use of human induced pluripotent stem cells (iPSCs) and neural organoids. Human iPSCs are adult cells that are reprogrammed into an embryonic stem

cell-like state. These cells are described as 'pluripotent', meaning that they have the ability to differentiate into all of the cells of the adult body. Human iPSCs can therefore be used to make brain cells (neurons) that can be used to test many things including the biology of disease.

Neural organoids (also known as brain or cerebral organoids) are artificially grown tissues that have some characteristics similar to the brain. They are often made from human iPSCs, and form three dimensional structures of many thousands of brain cells in comparison to the typical two dimensional iPSC cultures with relatively low numbers of neurons. Neural organoids are a relatively recent development, and have great potential as a non-animal alternative for answering certain research questions.

Why were they not suitable?

To fully understand the contribution of genes linked to neurodevelopmental disorders (NDDs) during development, It is generally accepted within the scientific literature that this should be carried out in an animal that has developed normally in utero. I am committed to using non-animal alternatives where possible and will explore the use of these to address research questions raised during this project. For example, it is anticipated that hypotheses will be generated during this project that can be directly tested using neural organoids. This will allow for the testing of whether NDD gene mutations affect neuron development in the same way in an intact animal in utero (in the womb) as in cultured cells in vitro (in the lab).

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 used power analysis to determine group sizes based on data from pilot studies that form the basis of this project. For most studies a sample size of 8-10 rats/mice is required per group. Breeding number estimates are based on annual returns generated during the pilot study.

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

I used the NC3Rs experimental design assistant to ensure that the minimum number of animals are used while meeting the scientific objectives 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?

Breeding strategies will typically involve breeding heterozygous females with wild-type males. Animals will be bred in-house and kept on a 12 h/12 h light/dark cycle with ad libitum access to food and water. Following weaning up until the start of the experiment, knockout animals (e.g. FMR1 KO) and wild-type (no genetic mutation) littermates will be

group-housed in mixed-genotype cages. Animals will be selected randomly from a litter for use in each experiment. Breeding pair numbers will be continuously monitored to ensure minimal wastage of animals.

From the brain tissues taken neurons will be filled with markers during ex-vivo whole-cell recordings, allowing the same cells to be used for physiology and morphology experiments, thus reducing the number of animals required.

Pilot studies have been carried out to help generate realistic effect sizes for power calculations.

Computer modelling of neurons will be used to generate testable hypotheses prior to experimental validation in 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.

Genetically-modified rats and mice will be used, these models have previously been shown to have no moderate or severe effects as a result of these mutations. Rats will be used primarily as they have larger brains than mice and exhibit a more complex behavioural repertoire, yet still retain many tractable characteristics amenable to laboratory research. A relatively small number of mice will be used to explore NDD models not yet available in rats (eg SHANK3).

Several of the models used are x-linked, meaning that syndromes linked to these mutations are more prevalent in males as compared to females. The result of this is that the majority of labs use male animals only in their studies. For this reason we will also prioritise male mice for physiological testing. However, where possible we will also endeavour to test female animals to determine the extent to which the phenotypes we observe are prevalent in both sexes. Where females cannot be used they will be culled at the earliest opportunity. Surgeries will only be carried out on a subset of animals, with the majority being used without undergoing this procedure.

Mice from the background strain will also be used to generate a dataset in mice that will allow for future use of mouse models of neurodevelopmental disorders .

Cardiac perfusions will be carried out under terminal anaesthesia. This is classified as a mild severity due to the use of anaesthetics.

Stereotaxic surgeries will be performed under sterile conditions to minimise the risk of infection. Analgesics will either be injected or given orally to minimise pain.
Absorbable sutures are also used to minimise discomfort. From past experience I have observed that animals recover well within one hour of surgery, behave normally within one

day, and are indistinguishable from animals that have not undergone surgery when used 2-3 weeks later.

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

Using animals in the first 2/3 of gestation does not allow the development process to reach completion and is not compatible with the research objectives of the project.

Models of the neurodevelopmental disorders I wish to study are not available in less sentient species, meaning that alternatives such as zebrafish or invertebrates are not currently viable. Furthermore, using rats and mice allows for hypotheses to be generated as to potential effects of the phenotypes observed in individual neurons In relation to behaviour.

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

Animals will undergo additional monitoring post-surgery, thus allowing for any issues to be picked up at an early stage. Animals are given analgesics prior to, during and after surgeries to minimise pain.

Animals will be culled humanely once experimental objectives have been achieved and will be used at the youngest age possible. Animals will be regularly handled to allow for habituation to the handling process and to reduce stress. Tube handling will be used routinely when handling mice.

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

I will adhere to the PREPARE, ARRIVE and LASA guidelines throughout the project.

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

I will routinely attend NC3Rs events and webinars and will regularly check information on the NC3Rs website. I have also signed up for the NC3Rs newsletter. I will also continue to seek advice from the Named People as to how advances in the 3Rs can be implemented throughout the project.

64. Using RNAs to investigate the mechanisms of skin repair

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

skin, repair, wound, matrix, epidermolysis

Animal types	Life stages
Mice	adul, 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?

Skin can be injured by a range of actions such as surgical intervention or unintentional trauma. Studies in mice have already shown that small wounds on their backs heal very efficiently when filled with wound matrix: the temporary tissue, which naturally replaces blood clots. The aim of this project is to find out how the wound matrix changes and test if small RNAs can be used to change the process of wound repair. Furthermore, the identification of adhesive molecules that improve the formation of wound matrix could help wound healing and alleviate devastating disorders such as epidermolysis bullosa (EB), which is characterised by fragile skin that blisters and tears easily.

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 a major barrier protecting the body, skin is often damaged. While healthy skin repairs well, many underlying health conditions such as diabetes may result in persistent ulceration or large-area wounds with impaired healing. More efficient healing of ulcerated wounds can be achieved if we learn more about the adhesion mechanisms controlling wound matrix formation. Wound matrix consists of many different cells that produce adhesive molecules responsible for efficient skin repair. Much of these functions rely on

adhesive properties of the molecules by which cells are held together in the wound gap. In addition, the cells themselves have adhesive molecules that allow them to migrate to close the wound gap. These molecules are also crucial for keeping the skin layers together and when they lack functionality, it can lead to dissociation of skin layers, known as blistering. Thus, the identification of new adhesive molecules that improve the formation of wound matrix could help wound closure and alleviate blistering diseases like EB, a life-threatening genetic disorder with no cure.

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

1. Publication in scientific journals

2. Identification of new molecular mechanisms controlling adhesion in skin.

3. Testing new potential novel therapies for wound repair.

4. Development of wound assays in vitro with potential application for the replacement animals.

5. Proof of principle that the inhibition of small RNAs - tiny switches that help control which genes are turned on or off - can restore adhesion in EB.

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

1. The scientific community in academia and industry by knowing how adhesion works in mice and cultured human skin cells.

2. Patients will eventually benefit from the development of improved wound treatments.

3. A new avenue for epidermolysis bullosa (EB) treatments may open up for further clinical research, and patients may eventually benefit.

4. Animal welfare will be enhanced through the development of non-animal methods to evaluate the effectiveness of tissue regeneration treatments using wound matrices.

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

To disseminate the output of this project, I will participate in professional meetings, collaborate with other researchers in wound repair field. I will communicate with other mouse-based project leaders in the field about the use of the experimental methods to evaluate the effectiveness of wound treatments using wound matrices. Publications, social media and press releases will also be used.

Species and numbers of animals expected to be used

- Mice: 880

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 remain the main model of impaired (or improved) cutaneous wound healing in vivo for three major reasons:

1. Genetically modifiable and genetically controlled breeding lines make it possible to test specific molecular mechanisms by turning different genes on and off.

2. High resemblance and conservation of mechanisms of skin repair between mice and other mammalian species, including domesticated animals and humans.

3. Short reproductive cycle of mice makes the project aims feasible to achieve within the terms of typical funding and the license.

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

1. Mice will be bred to achieve the genetic type needed for experiments by deletion of specific genes.

2. After pain killers and anaesthetics are given to mice, they will be shaved to expose skin on the back. Then, each mouse will receive two small (6mm) wounds on the back skin.

3. As an option, wounds will be treated with a simple dressing pre-soaked with oligonucleotides. These are short, custom-made strands of nucleotides, the basic building block of nucleic acids which all living beings need. If the dressing doesn't stay on, the oligonucleotides will be injected near the wound site once, after wounding has been administered.

4. All animals are expected to make a rapid recovery from the anaesthetic within two hours. Animals that fail to do so or exhibit signs of pain, distress or of significant ill health, which is rare, will be humanely killed.

5. EB blistering mouse models will be genetically modified so that the blistering disease will eventually heal.

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

Though the incidence of adverse effects during surgery is very rare, the animals will be monitored regularly for any signs of infection or abnormal behaviour. If any of these are indicated, the animal will be humanely killed.

The treatments to be applied or injected in to the wounds are not anticipated to cause any adverse effects to the animals. They may accelerate wound healing, or they may impair wound healing, however this should not produce any additional discomfort for the mice.

EB mice will only be observed for blister development on their ears, footpads, and tails, and may experience discomfort associated with redness and irritation of the skin covering these parts of the body. As soon as the symptoms appear, they will be scored as described in the protocol, and the animals will be humanely killed. We expect to alleviate the blistering of the EB mice by crossing them to mice with genetically deleted microRNA-29, a small, non-coding RNA molecule that plays a crucial role in regulating gene expression.

Animals will be kept warm and carefully monitored during the procedure and in the recovery period afterward.

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 mice which are genetically modified will experience mild severity, and mice which experience wounding moderate severity. We expect to alleviate the blistering phenotype of the EB mice, which has a moderate level of severity, by crossing them to microRNA-29 deletion mice which has a mild level of severity mild.

What will happen to animals used in 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 must use animals in this study because testing how different types of skin cells interact during wound healing, as well as detachment of epidermis from the dermis in the EB model, must be studied in the complete physiological setting. That way, we get an accurate picture of this process. We cannot fully use human cells for these experiments because we would not be able to monitor the physiological whole-body response to wounding.

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

Human skin cells isolated from patients' skin biopsies could be used to partially answer the aims of the project. Using the information from mouse wound matrix, we will test the conditions to reconstitute the matrix in vitro using human cells to develop novel replacement models. While it is not possible to fully replace the mouse model, the human in vitro model will help to reduce the number of animals in future research not only for our projects but also for other researchers in the field.

Why were they not suitable?

There is not enough known about the adhesion molecules regulated by the microRNA-29 oligonucleotides to make the full use of an in vitro system. We need to study wound matrix adhesion in the microRNA-29 deletion mouse first. Based on this, we will develop the human in vitro model of wounds modifiable by the oligonucleotides.

While the EB model exists in human skin equivalents, we cannot test the genetic rescue of the disease without using genetically altered 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?

By consulting with expert colleagues, I have refined the experimental design. I will also test potential therapeutic treatments in cell culture models of wounds before we work with animals which will both reduce the risk of adverse effects and reduce the number of animals needed. To plan for our animal work, I have consulted a statistician to establish the minimum number of animals required for each study. Also, where possible, I will use two wounds per animal to reduce the number of animals required, using wounds collected from both male and female mice.

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

1. I used the Experimental Design Assistant (EDA), a free online tool from the NC3Rs to accurately reduce the number of animals being used in this project.

2. Control mice will be the wild type littermates (in microRNA-29 knock-out experiments) or non- specific control oligos applied to one wound vs the other on the same animal. This will maximise the data output from the mice I use and will also minimize the variability.

3. I have data from my previous work to help to determine the minimal number required per experimental group.

4. I developed parts of the protocol for the replacement of mouse wounds with human 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 will use a commercial genotyping service to cut the time for genotyping PCR to ensure efficient breeding. My group uses computer modelling to analyse wounds and we also receive and share wound samples for sectioning and imaging 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.

Mice are the least sensitive mammalian species that can mimic a systemic wound healing response in humans. In addition, the only genetically modified models we are working with are available in mice.

We have expertise in experimental and surgical work with these models and always use the most refined experimental design and employ sharing of tissue from each animal used whenever possible.

The use of anaesthesia and analgesics are used where appropriate to minimise discomfort and stress to the animals. All team members will be aware of the need to minimize the impact on each animal and the necessity within each experiment to define humane endpoints.

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

Unlike mammals, lower organisms commonly used in tissue regeneration studies such as the fruit fly lack all microRNA-29 molecules. Thus, only mammalian, namely, mouse, skin regeneration model can be employed to study microRNA-29 function in wound healing.

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

Skin inflammation is an integral part of healing, though skin irritation is minimal and the inflammatory symptoms are transient. We shall use analgesia and the aseptic technics to minimise the inflammation.

We are using the least severe blistering model to test the possibility of genetic rescue of the blistering symptoms. The Junctional Epidermolysis Bullosa mouse model (JEB) we're using, where outer and inner layers of skin are separated, has less severe symptoms compared to both other mouse models and the actual disease in humans. It is the most suitable model for the specific purpose of the study which allows us to focus on the specific genetic mechanisms involved in the condition and potential treatments, despite the mouse model not fully replicating the severity seen in human cases of JEB.

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

I will be keeping up to date with wound healing literature and have designed studies using appropriate controls. I also used the smallest group size per experiment to effectively test effects on wound matrix formation. Wounds can be treated as ‘independent’ because previous studies have shown greater variability in wound healing between wounds within a mouse than between litter mates.

The breeding of mice developing a mild blistering has been performed and published by academic labs and commercial breeders.

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

The animal facility regularly conducts 3Rs workshops and send me the most updated 3Rs information. This information is also updated on the Establishment SharePoint, which I check regularly.

I will also keep up with the current literature reporting the development of alternative models of wounds and consult the local 3Rs managers and will be supported by the NVS advice at every stap of the project.

65. Smart nanomaterials for solid cancer therapies

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

nanomaterials, cancer, therapy, surgery, metastasis

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 aim to mechanistically understand the role of 'smart' nanomaterials in improving conventional cancer treatments in early and late-stage cancer of solid tumours. Furthermore, we seek to understand the influence of hypoxia, immune exclusion, tumour heterogeneity, and cancer invasion in preventing the therapeutic outcome of conventional treatments and strategies to overcome these barriers using 'smart' nanomaterials

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?

Solid tumour cancers, which account for approximately 90% of adult human cancers, manifest in various body regions such as the breast, lungs, brain, and prostate. Despite advancements in conventional treatments like surgery, chemotherapy, and immunotherapy, large or metastasised solid tumours remain challenging to cure completely. Hence, there's a critical need for innovative treatment strategies to augment these conventional approaches. Nanotechnology-based drug/protein/gene delivery

systems present promising avenues for tailoring cancer therapies to achieve robust antitumour activity. Among these, smart nanomaterials excel in precisely delivering therapeutic payloads to tumours or associated organs, inducing cancer cell death while sparing healthy tissues. Continued research into developing smart nanomaterials capable of targeting specific cancer cells and releasing cargo could significantly enhance clinical cancer treatment outcomes. Understanding how various parameters like composition, size, and surface properties affect the behaviour of smart nanomaterials in vivo is crucial for their effective use as gene, drug/vaccine carriers. Our research has yielded a library of smart nanomaterials designed to deliver therapeutic payloads, aiming to improve conventional treatments such as surgery, chemotherapy, and immunotherapy.

Despite advancements in surgical techniques, local tumour recurrence remains a significant challenge in solid tumour cancers, often leading to poor prognosis. To address this, we will develop post-surgical tumour recurrence models in mice to investigate the efficacy of nanomaterial treatments in preventing or inhibiting tumour recurrence and elucidate the underlying biological mechanisms. This research will inform the design of novel treatment strategies using nanomaterials for clinical application. Additionally, we will explore the potential of nanomaterial-based therapies in preventing perioperative immunosuppression and reducing cancer recurrence rates in cancer surgery patients using mouse models of spontaneous lung metastasis and surgical stress. Furthermore, our study will investigate the combinatorial effect of nanomaterial-based chemotherapy, immunotherapy, or chemoimmunotherapy in peritoneal carcinomatosis of solid tumours such as colorectal, ovarian, pancreatic, or lung cancer. By addressing critical barriers like hypoxia, immune exclusion, tumour heterogeneity, and cancer invasion, we aim to enhance the therapeutic outcomes of conventional treatments using smart nanomaterials.

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

At the end of this project, we will be achieving these key outputs.

New information from this project:

Short term: Obtaining fundamental knowledge about the therapeutic efficacy and safety of smart 'nanomaterials' in various solid tumour cancer models. The knowledge obtained from this project will be disseminated to the public and other researchers working in the cancer field by attending local and international conferences (poster and paper presentations), giving guest lectures etc. Furthermore, the strategies and technologies or tools developed based on the 3Rs (Replacement, Reduction, and Refinement) concept in this project will be conveyed to the public and research community by presenting or discussing in conference or forum such as The European Partnership for Alternative Approaches to Animal Testing (EPAA) annual seminar and a pint of science.

Midterm: Under the ARRIVE guidelines, the research findings from this project will be published in top peer-reviewed journals. In addition, the research findings will support obtaining future research grants and fellowships to support and conduct advanced preclinical or clinical studies.

Long-term: We are anticipating that the outcome of this preclinical research work will bring alternative treatment options for advanced solid tumour cancers in patients undergone pre or post-conventional treatments. For instance, we have successfully developed a clinical-grade polymeric nanovaccine for head and neck cancer, showcasing remarkable therapeutic efficacy in a subcutaneous mouse model of head and neck tumours. Building on this success, our next step involves testing this vaccine in a more advanced and clinically relevant mouse model—the postsurgical tumour recurrent model. The new

information from the current work will help in the clinical translation of novel smart nanomaterials.

Furthermore, the research output will support patent filing and could lead to the development of commercial products for clinical or pharmaceutical purposes.

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

Primary benefits

1. Dissemination of novel information by publishing papers in international journals and presenting our findings at scientific meetings and conferences. This will benefit the research community as the outcomes of this project will provide significant advances in knowledge in the following fundamental areas of nanomedicine in improving the treatment outcome: understanding the mechanisms of how smart nanomaterials deliver and release the drug in the tumour microenvironment; the role of the immune system on the antitumour activity and the tumour microenvironment complexity in metastasis, therapy resistance and hypoxia promoting tumour recurrence post-conventional treatment.

2. The research community will benefit from these interdisciplinary research methodologies that we develop and plan to make available for use by others. These will be validated by our work and will enable others to use them in their discovery-led research or research-led innovation. For example, the multi-omics analysis of tumour tissue post-treatment will be critical for others to design their preventive drugs for human cancer disease post-conventional treatment.

Secondary benefits

1. Pharmaceutical industry

The results generated from this project will be of significant interest to the pharmaceutical industry concerning formulating novel drugs or gene material delivery systems for cancer treatment. Our work will allow pharmaceutical companies to mass produce effective drug or gene formulations that will provide alternative treatment solutions for current clinical problems in cancer. Since our nanomaterials mimic the existing biomaterials approved by the Food and Drug Act (FDA) USA or UK, it is easier to get them approved for clinical applications in cancer. Even more, our novel smart nanomaterials can be used for pharmaceutical applications in various diseases other than cancer.

2. Government, society and economy

Solid tumour cancers, including those originating in the breast, lung, colon, and skin, represent a predominant global health concern. While conventional treatments like surgery, chemotherapy, radiation therapy, and immunotherapy exist for managing these cancers, they come with significant drawbacks such as toxicity, limited long-term efficacy, and poor bioavailability. The current approach of employing smart nanomedicine for cancer treatment holds the potential to enhance patient survival rates by improving the outcomes of conventional treatments and mitigating their toxicity and side effects. Moreover, the smart nanoparticle system may reduce the necessity for repeated treatment regimes, potentially leading to substantial cost savings for cancer treatment and services within the NHS. This innovative strategy not only enhances treatment effectiveness but, more crucially, aims to alleviate the suffering of individuals impacted by the progressive tumour. In contrast to certain traditional therapies that may harm healthy tissues, our smart nanoparticle system stands out with its highly targeted and precise approach, offering

potentially superior benefits compared to other cancer therapy methods. This distinctive feature positions our research at the forefront of innovation, dedicated to improving both treatment efficacy and the overall quality of life for cancer patients.

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

Based on the outcome of this work,

we will approach clinicians and pharmaceutical industry partners for collaboration to further take these nanomaterials to the market. The study findings will be published in a peer-reviewed journal(s) (in accordance with ARRIVE guidelines).

Furthermore, Dissemination of results and knowledge to the scientific community via scientific papers and meetings. Putting novel resources onto searchable databases and repositories so that others can access and use them.

Based on the knowledge obtained from these studies, more focus will be put forward on finding alternatives or replacements for animal research. More efforts will be put towards developing technologies such as organ-on-a-chip or 3D bioprinting.

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 use adult mice throughout the project as they are an excellent model for cancer research. The tumours grown in mice mimic those of human cancer patients. Studies of cancer in mice mimic the complex way tumours grow and spread in people with cancer. Mice can be easily genetically altered to allow us to study the genetic causes for poor or improved treatment outcomes in cancer and reproduce tumour types which naturally occur in humans in the correct tissues and body systems. Furthermore, it gives us the advantage of a rich suite of genetic strategies to target specific cell populations in the tumour and immune system either to specifically record their activity, manipulate their activity, or alter their gene expression. These strategies are essential to allow the rigorous analysis of the underlying mechanisms involved during smart nanomaterial-based cancer treatment. In the 3Rs viewpoint, adult mice are a better replacement for more complex or higher-order sentient animals. Furthermore, meaningful data with a statistically appropriate number of mice will minimize the need for additional animal subjects, aligning with the principle of Reduction in the 3Rs perspective.

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

Mostly, the mice will be injected with cancer cells either subcutaneously or orthotopically (breast or skin) to develop cancer animal models. Sometimes, healthy (non-tumour) mice will be used for studies such as assessment of the maximum tolerated dose of the smart nanomaterial or collecting tissues for in-vitro experiments. For pharmacokinetics analysis, we will take blood from the superficial blood vessels in live mice or via heart puncture under deep terminal anaesthesia, ensuring that the mice do not recover from anaesthesia.

Whereas biodistribution of smart nanomaterials will be assessed using a live animal imaging system with or without anaesthesia. For the post-surgical tumour recurrence model, the developed tumour mass will be surgically removed under anaesthesia and later monitored for metastasis. Furthermore, the metastasis (peritoneal carcinomatosis) mouse model will also be developed by injecting the cancer cells directly into the animal via intraperitoneal injection. This will mimic the human peritoneal carcinomatosis condition that occurs when the solid tumour cells disseminate into the peritoneal region. Most of the experiment will involve wild-type mice, however, depending on the specific gene of interest, genetically altered mice will be employed for the study.

Depending on the nanomaterial characteristic feature, the following route of administration of nanomaterials or cancer cells will be used: subcutaneous, Intraperitoneal, Intravenous, Intranasal (e.g. for nanoparticle delivery to lung and brain), peritumoural, Hock injection (e.g. to investigate lymph node-to-lymph node trafficking of nanoparticles), intratracheal intubation (e.g. for nanoparticle delivery to lungs), and oral administration.

Sometimes, mice will be euthanised using the schedule 1 procedure early to provide tissue for cell characterisation from cancer tissue or associated tissues like lymph nodes or spleen. However, in other cases, mice will be kept alive until they reach either the scientific or humane endpoint, depending on whichever comes first. For example, during a tumour mice survival study, if a mouse's tumour size reaches 1000 mm3 or if it shows signs of distress such as maximum weight loss upto 15%, it will be euthanised according to humane endpoint criteria. On the other hand, if 100% of mice in the control group have reached the humane endpoint, then the decision to euthanise the treatment group after a certain period will be taken, even if they do not meet the humane endpoint criteria yet. This approach ensures that the study captures relevant data while also considering the welfare of the animals involved. During this time, we will use a variety of non-invasive tests to measure tumour volume (using a Vernier calliper), metastasis (using a live animal imaging system), and body weight (using a weighing machine) to examine the therapeutic effect of our smart nanomaterials post-conventional treatment.

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

We will take careful measures to minimise potential impacts and adverse effects on mice. The surgical procedures may cause short-lasting pain, which will be mitigated using analgesia. Following recovery, there are minimal adverse signs. Our pharmacokinetic testing or safety assessment is non-invasive and has no adverse effects. We do not expect the non-viral genetic manipulations using nanomaterials to cause harmful phenotypes. Importantly, our experimental design does not anticipate adverse outcomes such as weight loss (more than 15%), extreme tumour necrosis or ulceration (deep), or infections. However, in the unlikely event of any unforeseen complications, we will perform Schedule 1 euthanasia. Additionally, suppose there are signs of pain or discomfort, due to an open wound on the tumour caused by scratching, lack of evidence of healing (e.g., no tissue granulation or skin contraction around the ulcer for more than 48 hrs), and a consistent wet discharge (such as pus, clear serum, other fluids, or blood), we will also perform Schedule 1 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)?

The expected severities are moderate in all animals, except for GAA animal breeding which is mild.

Animal type	Severity	Number of mice	Proportion (%)
Adult mice	mild	1000	22.5%
Adult mice	moderate	3500	77.5%
Adult mice	severe	0	0

What will happen to animals used in 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 seek to understand the influence of hypoxia, immune exclusion, tumour heterogeneity, and cancer invasion in preventing the therapeutic outcome of conventional treatments and strategies to overcome these barriers using 'smart' nanomaterials. All these barriers can only be observed in the tumour tissue of the whole organism and hence they can only be studied in whole animals and tissues taken from animals. Rodents are generally accepted as being good models for these aspects of human tumour physiology. It would be unethical to use patient subjects in these experiments.

Although we will still need to perform the assessment for cytotoxicity, intracellular uptake, and anticancer activity in in vitro models. We shall also use ex vivo preparations to assess the effects of antitumour immune modulations induced by smart nanomaterials. Additionally, we have replaced the use of animals in screening the library of nanomaterials using our 3D culture methods. For example, our use of the tumour spheroid-co culture of immune cells will allow us to test the therapeutic effects of 'smart' nanomaterial before the development of lead 'smart' nanomaterials for in vivo experiments.

Together this combination of ex vivo and in vitro work will act as a replacement, minimizing rodent numbers by only using effective and safe nanomaterials and removing risk from subsequent work on whole animals.

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

In vitro 2D and 3D cell, culture techniques will be used to understand the toxicity and therapeutic activity of the nanomaterials. Furthermore, we are developing an organ-on-chip tumour model using the microfluidic system or 3D-printed hydrogels to initially screen the library of nanomaterials.

Computer modelling will be used to screen the best nanomaterials based on the ex vivo in vitro analysis.

Why were they not suitable?

In vitro 2D and 3D cell culture using cancer and immune cell lines. While these can be useful for assessing anticancer properties of smart nanomaterials prior to use in vivo (and we use them for this), these cells cannot reproduce the complete physiological functions such as enhanced permeability and retention (EPR) effect, antitumour immune memory,post-surgical metastasis, the abscopal effect that we wish to study.

organ-on-chip tumour model. These have not reached the level where they can reproduce the mechanisms and interacting systems that we need to study to advance the field. Furthermore, it is difficult to bring in multiple factors present in the animal system into the organ-on-chip model.

Computer modelling. All good models require data to allow them to be realistically specified and hence useful as predicted tools. We simply lack sufficient data to enable this approach as a realistic way forward.

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 considered the range of experiments and manipulations that we need to perform to advance knowledge. Using information from our prior work or the work of others we can calculate the numbers we need to give definitive results that we can be confident will be reproducible by others.

Where effect size and variation are available or can be estimated from the literature, experiments will be planned based on power or 80% and at a level of alpha < 0.05. We shall use GPower software to perform power calculations. If necessary for more complex experimental designs we shall use the NC3Rs experimental design assistant.

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

We used the best estimate of effect size based on pilot data or published literature to inform our power calculations. Furthermore, we will use online reference tools such as NC3R's experimental design assistant for experiment group design 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?

We shall ensure all personnel are highly trained and skilled so that very few experiments will fail for technical reasons.

Pilot studies will be performed to get good estimates of effect size, and feasibility, and ensure that all technical problems have been anticipated and solved. Should our program demonstrate that our estimates of effect size are wrong (either too big or too small) we shall reperform the power calculations to ensure we arrive at a rigorous outcome. If it appears that the effect size is much smaller than anticipated for a particular experiment,

we shall consider abandoning that part of the program. We shall routinely harvest tissue to enable biochemical, genetic, cellular and morphological analyses and share this tissue with our collaborators. Using bioluminescence imaging (assessing metastatic lesions) as a longitudinal measure of the animals will reduce the number of animals needed while each animal will only be mild to maximally (in sum) moderately affected.

We shall use efficient breeding strategies to minimize the number of animals that are bred. In some cases, we may have to maintain lines as heterozygotes meaning that animals (which do not carry the gene of interest) cannot be used. Where at all possible such animals will be used for control comparisons giving the benefit of identical genetic backgrounds, however, this means that we will unavoidably breed more animals than we will use in procedures.

Furthermore, the number of mice per group can be reduced by combining two or three experiments. For example, the tumour burden, body weight measurement, and survival rate of the animals can be performed as one experiment with n=8, instead of multiple experiments with n=13 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.

Rodents are a well-established model for studying cancer treatment and its relative outcome. They are also used to examine mechanisms of antitumour activity and the effects of the immune system on treatment outcomes using nanomaterials more generally. This provides robust literature and a wealth of potential data, obviating the need to repeat past findings. Rodents in general are seen as a simplified and experimentally tractable model for far more complex mammals such as humans.

Wherever possible, metastasis progress, nanomaterial tracking, and biodistribution could be achieved using non-invasive methods such as live fluorescence animal imaging to analyse nanomaterial biodistribution in the tumour. More invasive procedures might be used only when there is good reason (from the non-invasive experiments) to expect mechanistic insight. Moreover, the subcutaneous as well as orthotopic tumour mice models will be used during this project and they may not suffer any pain or discomfort due to the tumour growth since the location of the tumour does not hinder the day-to-day activities, movements, and consumption of liquid or solid foods. However, to avoid tumour scratching by mice, soft bedding materials such as paper-based bedding or tissue paper may be provided in the cages, as these have been shown to reduce the likelihood of tumour damage. Additionally, regular monitoring of the mice for signs of scratching or irritation may be conducted, allowing for prompt intervention if needed.

Moreover, the mouse model of surgical tumour resections might provide a prolonged timeline to investigate metastasis in mice. This model entails short-term mild to moderate pain or discomfort, which may be mitigated by providing either proper analgesics or pleasant environmental conditions such as gel foods and soft bedding. For this project, we

shall also utilise the peritoneal carcinomatosis mouse model due to its relevance in studying cancer progression and treatment. This model involves minimally invasive procedures, such as the injection of cancer cells into the peritoneal cavity, which minimises pain, suffering, and distress to the animals. Mice are easily monitored throughout the study, allowing for timely intervention if any signs of discomfort arise. We may use ex vivo tissue as much as possible to study the immune modulatory activity of nanomaterials. This may enable future in vivo experiments to be appropriately designed to minimise animal usage. All substances will be administered as per the LASA guidelines.

Especially, footpad injection is a widely used method for immunisation in mice due to its well- characterised lymphatic drainage and ease of administration. However, it is limited by using only hind feet for immunisation, as mice primarily use their forefeet for food handling. Additionally, footpad injections can cause inflammation and swelling at the injection site, leading to pain and distress since feet are weight-bearing structures. Hence, we might perform the injection into the hock, a non-weight- bearing area just above the ankle.

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

We wish to examine these phenomena in a mammalian system to have maximum applicability to the human condition. By choosing mice, we have chosen a model that has the lowest sentience.

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

We have considerable experience with in vivo imaging methods and have considerably refined them already over the past 2-3 years. We may implement standardised post-operative monitoring methods to ensure that outcomes and adverse effects from our surgical procedures are carefully assessed. This information may be used to optimise post-operative care and pain management.

We shall use rigorous aseptic techniques, including the use of sterile gloves and instruments to protect against infection of the animals. Additionally, assistance from a surgical unscrubbed person may be utilized to support mostly handling non-sterile equipment and materials, preparing the surgical area, assisting with anesthesia administration, recording data during the procedure, and providing post- operative care to the mice.

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

We shall take careful note of advice from collaborators and researchers highly experienced in these methodologies to ensure that we refine our experiments as much as possible. We shall follow the Laboratory Animal Science Association guidelines on performing aseptic surgery to ensure the best practice for the recovery surgery involved in all surgical procedures. For developing tumour models, the guidelines by Workman, P.,et. al., Br J Cancer 102, 1555–1577 (2010) will be followed to ensure ethical and scientific rigor.

To ensure effective and rigorous reporting of our results, we shall write papers according to the ARRIVE guidelines which are recognised as providing excellent transparent standards for reporting of research using animals and were developed by the NC3Rs.

We will follow NC3Rs guidelines for breeding genetically altered (GA) mice, which emphasize minimizing suffering through careful monitoring, strategic planning, humane endpoints, refined husbandry techniques, and consideration of welfare impacts.

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

We receive regular communications from NC3Rs and Laboratory Animal Science Association (LASA) via a newsletter, colleagues who advise on best practices, and the NACWO, NTCO, NIO and AWERB at our establishment. We consult the NC3Rs and LASA website to keep abreast of current best practices.

66. Searching for potential cures for traumatic injuries to the 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

Axon regeneration, Visual function recovery, Traumatic optic neuropathy, Optic nerve crush, Neuronal survival

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 central aim of this project is to understand the reasons for poor functional outcomes in patients with injuries to the central nervous system (CNS), in particular, traumatic optic neuropathy (TON). This project will (1) test genes, proteins, or novel drugs for their ability in promoting the regrowth of nerve cells and their processes to (2) reconnect with their original brain targets for improved visual functional recovery in a mouse model of optic nerve crush (ONC) injury. It will also (3) understand the mechanisms of axon regeneration and thus the knowledge acquired in this project can be applied for the design of novel therapeutics for other traumatic injuries to nervous system, such as spinal cord injury (SCI), traumatic brain injury (TBI) and peripheral nerve injury (PNI).

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 central nervous system (CNS) are often devastating which results in catastrophic loss of sensory and motor functions. These functional impairments are usually

permanent and irreversible which leads to life-long disabilities in the patients. Currently, there are still no effective interventions to improve the functional outcomes in these patients. The reason for the permanent functional deficits after CNS injuries is largely attributed to the regeneration failure of the adult brain cells. After traumatic injuries, these adult brain cells lose the ability to regrow their processes, namely axons, and thereby the connections between these brain cells and their original muscle targets are completely lost. More importantly, the myelin sheath, the insulator enwrapping the processes of the brain cells, breaks down after CNS injuries. For better conductance of the brain cells, the myelin sheath has to reform and enwrap the processes of the brain cells, after the regeneration of axons, a process known as remyelination. However, for some unknown reasons, this process is unlikely to occur so the processes of brain cells remain exposed (i.e. unmyelinated) even if they can be successfully regenerated, leading to poor nerve conduction and limited functional recovery after CNS injuries. Therefore, it is of utmost importance to identify key factors (genes, proteins, or novel drugs) that (1) boost the ability of brain cells to regrow their processes and (2) promote remyelination for improved functional recovery in patients with CNS injuries.

In contrast to the brain cells in the adult CNS, nerve cells in the peripheral nervous system (PNS) have some ability to regrow their processes and reconnect to their original muscle targets for functional recovery after injuries. However, the growth rate remains extremely slow (1-2 mm/day). Therefore, it might take months or years for the nerve cells to fully regrow the processes if the patients suffer from proximal peripheral nerve injuries (e.g. brachial plexus nerve injury) which require long-distance axon regeneration. By the time when the nerve cells finally reach their original muscle targets, they fail to re- establish functional connections largely due to muscle wasting or muscle atrophy. Therefore, we aim to identify key factors (genes, proteins, or novel drugs) that accelerate the regrowth of nerve cells' processes so that reconnection of the original muscles can occur before muscle wasting.

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

Upon completing this project, our aim is to uncover the crucial elements that promote the regrowth of nerve cell connections (axon regeneration) and the restoration of the protective layer around these regenerated connections (remyelination). The discoveries made from this project should significantly enhance the recovery of function following traumatic injuries to the nervous system, such as peripheral nerve injuries, traumatic optic neuropathy, and spinal cord injuries. These crucial elements could range from genetic materials like DNA and various types of RNA, to proteins, and epigenetic modifiers that influence how genes are expressed. Additionally, we will screen for new drugs that potentially boost axon regeneration and functional recovery. We will investigate whether these factors (genes, proteins or drugs) can effectively stimulate axon regrowth and remyelination, ultimately expediting functional recovery after nerve injuries. Furthermore, we will delve into how these factors facilitate a faster regrowth of nerve cell connection and the restoration of the insulating layer around them. By pinpointing these critical factors, we hope to pave the way for the development of innovative therapies that can be readily deployed in clinical settings following successful clinical trials.

All the results will be published in the form of academic journals, and the data generated from this project will be available to researchers who want further studies upon reasonable request to the project licence holder. All the high-throughput sequencing data that is possibly derived from this project will be deposited to Gene Expression Omnibus with an accession number provided by NIH.

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

We believe that individuals suffering from various nervous system injuries, such as peripheral nerve injuries (like brachial plexus nerve injuries), traumatic optic neuropathy (a condition where the optic nerve is damaged, leading to vision impairment), traumatic brain injury, stroke, and spinal cord injuries, could benefit greatly from this project. Injuries to the nervous system often result in catastrophic loss of function because nerve cells struggle to reconnect with their intended targets. Our project aims to address this issue by seeking powerful growth stimulators to encourage nerve cells to regrow towards their original targets, thereby restoring lost functions. We are optimistic that our efforts will offer potential solutions for better clinical outcomes for patients with all types of nervous system injuries.

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

We are actively seeking collaborations both within the UK and internationally. The applicant has established connections with researchers worldwide, including experts in bioinformatics, experienced pharmacists, and neuroscientists. Together, we arestriving to understand why nerve regeneration fails after injuries to the nervous system. We regularly participate in local and international conferences to share our exciting discoveries and seek opportunities to collaborate with clinicians for potential clinical trials. Our findings will be published in esteemed academic journals, possibly in open-access formats to reach a wider audience. We will also work closely with our institution's press team to ensure that our results are communicated to the public, particularly to patients with nervous system injuries, so they can benefit from our latest findings. Additionally, we will share our results with various patient focus groups across the UK, including but not limited to Fight For Sight, Glaucoma UK, Spinal Research Trust, Brain Research UK, and Wings for Life.

Species and numbers of animals expected to be used

- Mice: 3200

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, adult mice aged between 8 to 12 weeks will be used to create our mouse models. These models mimic injuries to the optic nerve after traumas in patients (i.e. traumatic optic neuropathy).

They are widely recognised as valuable tools for testing how well treatments work in improving function. Assessment of visual function can only be done on mouse models of optic nerve crush (ONC) injuries. We will use some established tests to measure the visual function of the injured mice after treatment. Since the functional problems in this model are similar to humans, any progress or improvement we see in the mice may translate directly to human patients.

In adults, nerve cells in the central nervous system (CNS) completely lose their ability to regrow their connections and processes after traumatic injuries. However, in younger mammals, the CNS displays some abilities regrowth in response to injuries. Prior research comparing young (postnatal to 8 weeks old) and adult CNS in mice has shown this stark

difference in the regeneration ability. Since most patients with nervous system injuries are adults, studying adult mice, which allow the research to perform behavioural assessments, is more relevant. Additionally, young mice are small in size which makes behavioural assessments challenging. Therefore, adult mice are the preferred choice for this study as they provide the most clinically relevant model.

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

Surgery:

Under general anaesthesia, mice will undergo surgery to induce damage to the optic nerve. Animals will be allowed to recover, and behavioural tests (see below) will be carried out to determine the degree of loss of function.

Administration of growth modifier:

To evaluate whether a particular factor or drug can effectively promote the regrowth of nerve cells' processes (axon regeneration), or the reformation of an insulating layer for the nerve cells (remyelination) following injuries, the shortlisted agent will be administered via different routes of administrations (i.e. direct injections to eye or nerve, injections to the peritoneal cavity, injections to the blood vessels, injections to the fatty tissues under the skin, and/or oral administration) to choose the best routes with maximal beneficial effects and bioavailability. While most of the routes can be done on restrained mice by a trained person (personal licence holder) with minimal discomfort to the mice, direct injections to the eye or nerve will be performed under general anaesthesia.

Assessment of axon regeneration:

For some experiments, a fluorescent dye will be directly injected into the nerve or eye to specifically label the regrowing processes before the mice are humanely killed. The procedure will be performed under general anaesthesia by a trained person (personal licence holder).

Animal behavioural assessment:

Visual functional recovery will be monitored weekly starting from week 3 or 4 post-injury, for a maximum period of 12 weeks post-injury (allowing sufficient time for possible regrowth of nerve cells and reconnection to their original targets in the brain). All the behavioural assessments for visual functions will be conducted on freely moving mice, except pupillary light reflex on gently restrained mice for video capturing purposes.

Humane killing

At the end point of each experiment, mice will be anaesthetised and infused with tissue fixative before being humanely killed.

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

In our study, mice with optic nerve damage are expected to lose a significant amount of vision in the injured eye, as this is a typical and expected outcome in this animal model. Visual impairment should be achieved as we have to assess the improvement of visual functions after our interventions. The vision in the unaffected/uninjured eye is anticipated

to remain intact based on our previous assessments. We take measures to ensure the mice experience minimal discomfort, including providing systemic pain relief after surgery. While most mice show no signs of pain, a small percentage (less than 20%) may experience brief acute discomfort at the injury site, which typically resolves within 48 hours after the administration of systematic pain relief. In less than 10% of mice, they may develop acute cataracts which typically resolve within 24 hours after surgical procedures.

In exceptionally rare occasions (usually less than 1% of cases), some mice may develop sustained cataracts or even eye damage due to the surgical procedures. In such cases, the mice are humanely killed using approved methods to maintain the integrity of the study's results.

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 with optic nerve injury, 100% of mice will suffer from moderate severity since optic nerve crush and direct injections to the eye have to be performed on all mice. Both of these are surgical procedures and thus moderate severity.

What will happen to animals used in 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 a mouse model of optic nerve injury to see how well gene or protein therapies and drug treatments work in helping mice regain their vision. While we start by testing these treatments in simpler lab setups, like cell cultures, these cell culture systems do not have the complex cell interactions found in a living organism, and do not allow functional assessment in a living organism. The mouse model provides a more realistic environment, allowing us to see the full effects of our treatments. More importantly, this well-established model helps us understand how potential treatments might restore function, which is crucial for developing new therapies for patients with nerve injuries. By using this model, we can gather important information that simpler tests can't provide, bringing us closer to finding effective treatments for nerve damage.

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

We explored alternatives to using animals in our project. One option we considered was using special nerve cell lines, like Neuro2A or SH-SY5Y cells. These cell lines can help us study how different genes, proteins, drugs, or other factors affect nerve cell growth by measuring how long the nerve fibres (axons) grow. We also looked into using primary neurons, in which nerve cells are taken directly from mice, as another way to replace animal models.

Why were they not suitable?

Neuro2A and SH-SY5Y cell lines come from cancer cells. Therefore, these cells behave differently from normal cells, making them unlikely to be suitable for studying nerve cell growth, which is crucial for our research.

Primary neurons from mice can help us measure the growth of nerve cell connections, an important first step in recovering function. However, they cannot be used to show if the treatments improve vision after an injury, which is the main focus of our project.

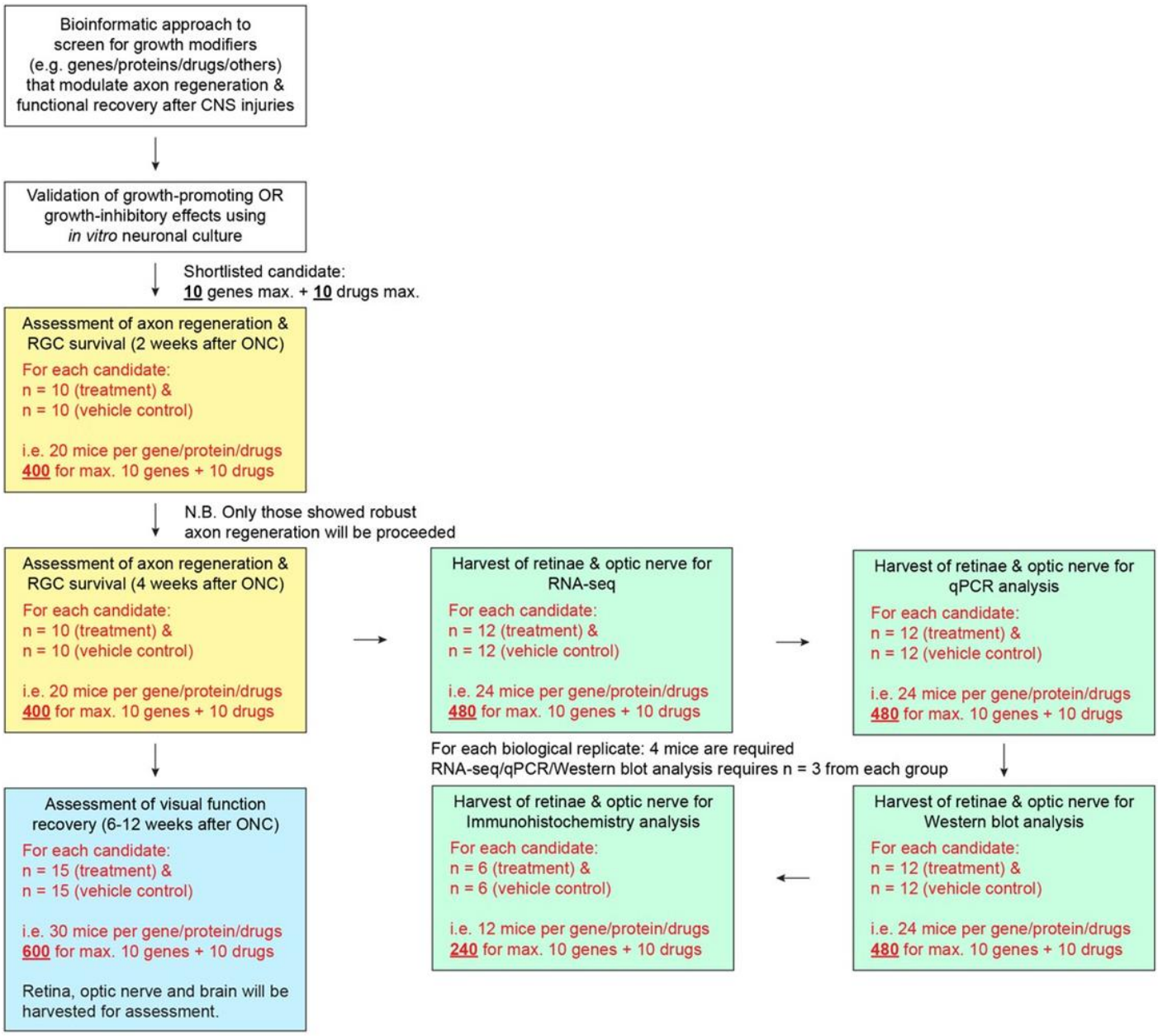
Primary cultures of another type of cell, oligodendrocyte precursor cells, can help initial screening of potential drug targets. However, they cannot show us how treatments affect the visual function behaviour of injured mice. To fully understand how well our treatments work in restoring visual functions, we need to use live mice. This makes mice essential for reaching the main goals of the current 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?

Please see the attached flow chart for the estimation of total mice used in the current project.



A total of **3,200** mice will be required for the entire project licence (3,080 mice + ~5% of buffer).

To assess the extent of axon regeneration after treatment of growth modifiers
To examine visual functional recovery after treatment of growth modifiers
To uncover the molecular mechanisms of the growth modifiers in regulating axon regeneration

Our project aims to find ways to help nerve cells in the central nervous system (CNS) regrow after injury. To start, we use a type of computer analysis called bioinformatics to identify potential growth modifiers, such as genes, proteins, or drugs, that might assist in this process. Then, we test these substances in cell cultures to see if they help or hinder nerve cell regrowth. Over the next five years, we hope to narrow down our list to about 10 promising genes and 10 potential drugs for further testing in mice.

Our project has three main goals:

1. To see how well these growth helpers promote nerve cell regrowth.

2. To check if they improve vision after treatment.

3. To understand how these growth helpers work at a molecular level.

To measure nerve cell regrowth, we will look at two time points: 2 weeks and 4 weeks after an optic nerve crush (ONC). The connections of the nerve cells in the CNS typically grow back slowly, and we can observe some regrowth within 1-2 weeks. By looking at 2-4 weeks after ONC, we can get a better idea of how much regrowth has occurred. In some cases, the regrowth might be so strong that we can see nerve cells growing into the brain by 4 weeks.

For each potential growth modifier, we will use 20 mice (10 for treatment and 10 for control). This means we will need a total of 800 mice to complete this part of the study for both time points.

Only the growth modifiers that show strong regrowth at 4 weeks will move on to the next step, where we assess visual function recovery. Based on our experience, 30 mice will be enough to evaluate visual recovery for each gene or drug, so we will need a total of 600 mice for this part of the study.

To understand how these growth modifiers work, we will use various molecular biology techniques including but not limited to RNA-seq, qPCR, Western blot, and immunohistochemistry (IHC). Each technique requires different samples, so we need separate batches of mice for each. For molecular studies, we need to collect tissues shortly after ONC to detect changes at the molecular level. Since only a small amount of RNA and protein can be extracted from the eye tissue, we will pool samples from 4 mice to create one biological replicate and will need 3 biological replicates for both treatment and control groups for each molecular biology experiment. Additionally, 6 mice will be used for histological analysis for both treatment and control groups. In total, we will need 1,680 mice to understand the molecular mechanisms of the growth modifiers (10 genes + 10 drugs; see the attached flow chart for detailed calculation).

In summary, our project will involve a total of 3,200 (3,080 + 5% buffer) mice over five years to achieve our goals of promoting the regrowth of nerve cell connections, improving vision, and understanding the underlying molecular processes.

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

We are using an online tool NC3R's Experimental Design Assistant to help us plan our animal experiments. This tool helps us figure out the right number of animals we need for our experiments, and ensures we use methods like randomisation and blinding to make our experiments fair and accurate. We have also consulted to our in-house statisticians to make sure we analyse our data correctly.

Our goal is to use the smallest number of animals possible while still getting scientifically valid results. This careful planning helps us get meaningful results without using more animals than necessary.

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

We started by using a computer-based method called bioinformatics to find potential substances (i.e. growth modifiers) that could help nerves regrow after injuries to the central nervous system (CNS). In the lab, we tested these potential growth modifiers in cell cultures to see how they affect nerve growth. Only those that showed significant positive

effects were chosen for further testing on animals. This method helps us reduce the need for animal testing while still finding promising candidates for promoting nerve regrowth.

In our animal experiments, we will first check if these growth modifiers can improve nerve regrowth after an optic nerve crush (ONC). Only those that show a significant boost in nerve regrowth will move on to the next step: assessing whether they improve vision and understanding how they work.

After confirming their effects on vision, we will see if the regenerated nerves connect properly with their original targets in the brain using the same mice. Only the most promising candidates will be studied further to uncover how they work at the molecular level.

For mice used in molecular biology experiments, we will collect various nervous system tissues for analysis. Some of these tissues, even those unrelated to the specific study, will be preserved for future research by others. This ensures that we make the most out of each animal's contribution to science.

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 mimic optic nerve damage in mice, we will gently compress the left optic nerve using smooth forceps. This procedure aims to disconnect the nerve cells between the eye and the brain, mimicking the visual impairment seen in patients with traumatic optic neuropathy. The surgery is straightforward and involves briefly compressing the nerve. Experienced researchers can complete the procedure in about 15 minutes. Wound healing occurs within days, with minimal scarring. After the injury, mice may experience vision loss in the affected eye, but their other eye remains functional. Any pain at the injury site will be managed with pain relief medication. Any acute pain condition will generally be resolved within 48 hours post-surgery, ensuring the mice experience minimal discomfort.

In most cases, drugs will be administered through injections into the peritoneal cavity, taken orally, injected into blood vessels, or injected under the skin. These methods are effective for delivering drugs with minimal pain and discomfort. Some drugs may need to be injected directly into the eye. In these cases, wound healing occurs quickly after surgery, with minimal scarring.

All surgical procedures will be conducted under general anaesthesia, and mice will receive pain relief medication for at least 2 days afterward to minimise discomfort.

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

In mammals, young nerve cells and other supporting cells have some capacity to regrow to meet the needs of a growing body. This regrowth is essential for the elongation of nerve cells and the myelination of expanding axons. In lower vertebrates like zebrafish and

Xenopus, optic nerve cells can fully regrow their connections after injury. Unfortunately, these models do NOT accurately represent the challenges humans face with nerve regeneration and myelination after CNS injuries. Only injury models in mice closely mimic the difficulties with regeneration and minimal functional recovery observed in human patients. Additionally, assessing functional recovery in animals under anaesthesia is impossible as they cannot perform the tasks under such condition. Therefore, mice serve as the most relevant model for studying nerve regeneration and functional recovery in humans.

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

Experienced personnel will administer general anaesthesia to ensure the mice are comfortable throughout the procedure. We will use a heat pad to keep them warm and maintain a steady body temperature. After surgery, the mice will recover on a heated pad and be closely monitored until fully awake before returning to their home cages. Pain killer (analgesia) will be given for at least two to three days afterward.

Following surgery, the mice may experience complete vision loss in the injured eye, but this doesn't affect their ability to eat, drink, sleep, or move normally. Since their other senses are crucial, they can still function well with lost sight on one eye only.

While most mice show no signs of pain after 48 hours, in extremely rare cases (less than 1%), if we notice typical signs of acute pain such as guarding, licking, biting, or restlessness; we will terminate the experiments and humanely kill the mouse by schedule 1 methods. This ensures their welfare and prevents unnecessary suffering.

Score sheet of animal suffering

Date and time of assessment: **Cage number:**
Assessor:

Category	Apperance	Score	Animal ID				
			1	2	3	4	5
Apperance	Normal and no sign of discomfort	0					
	General lack of grooming	1					
	Coat Staring, ocular and nasal discharge	2					
	Piloerection	5					
	Rash, pallor, redness, wound, ulcer	5					
Weight	Normal	0					
	Weight loss <10%	20					
	Weight loss = 10 -20%	50					
	Weight loss >20%	100					
Locomotor activity	Normal	0					
	Hyperactivity (circling, increased aggression)	5					
	Hypoactivity (hunched up, reduced explorative activity)	5					
	Reluctant to move, use of support, difficult to assess food and water	20					
	Ataxia, tremors	20					
	Seizure	50					
	Sign of coma	100					
Eye condition	Bleeding from the eye	50					
	Sustained bleeding	100					
	Clear sign of infections	50					
	Sign of cataract	20					
	Unresolved cataract	100					
	Abnormality in eye (immediate inform the PI)	20-100					
	Removal of eyeball	100					
	Total:		0	0	0	0	0
	Accumulative score:						

Humane Endpoint: If the mice reach the accumulative score of 100, they have to be humanely killed by Schedule 1 methods.

Monitoring guideline: The mice will be assessed daily for the first week post-surgery, then weekly for the rest of the entire course of experiment.

We will also implement a score system to grade the suffering of the animal daily for the first week post- surgery, and weekly until termination of experiments. The cumulative score of each animal will be assessed by our team members and the animal will be humanely killed once the threshold is reached.

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

We will follow the published PREPARE and ARRIVE guidelines for refining our experimental design.

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

We have subscribed to the newsletter from NC3R. We will constantly attend virtual conferences held by NC3R to keep ourselves well-informed about the advances in the 3Rs.

67. Regulation of the gut microbiome, immune and epigenetic landscape in healthy and non- healthy 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

Ageing, Hematopoiesis, Epigenetics, Microbiome, Frailty

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?

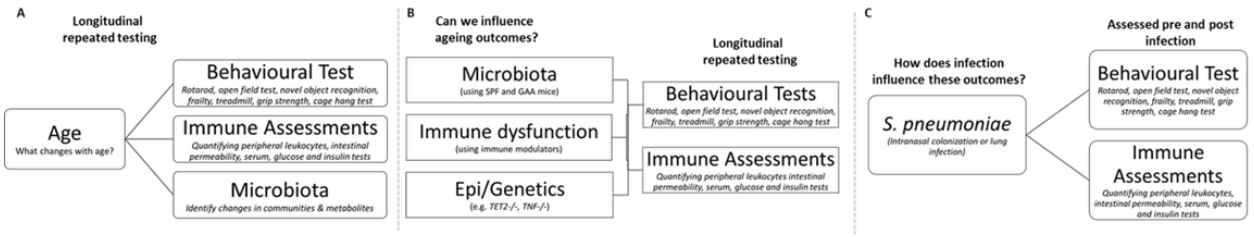


Figure 1. A) The aim of this project is to identify the underpinning mechanisms of a healthy vs. unhealthy ageing trajectory. We focus on repeated measures of mice over time and investigate how manipulating different factors (B) can change ageing outcomes. We manipulate the microbiota by performing colonization experiments, target ageing immune dysfunction with immunomodulatory drugs, and investigate how genetic knockout models (e.g. TNF KO and TET2 KO) change the ageing trajectory. We also aim to assess how infection (C) influences these outcomes by measuring them pre- and post-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?

Scotland, like most of the developing world, has an ageing population. Scottish individuals aged 65 and above currently make up less than one-fifth of the population, but account for nearly half of all health and social care spending. Over the next 20 years, the proportion of older adults is expected to grow by 30% posing additional changes on government finances. While this burden is often attributed to chronological age, it is actually age-related disease that challenges the health and social care system. The presence of one or more chronic condition (e.g. cardiovascular disease [CVD], cancer), infectious disease, cognitive decline and frailty are greater predictors of health care and social service utilization than age. To this end, an older adult in good health uses no more resources than a young person in good health. Understanding factors that contribute to these conditions in older adults is therefore a key priority for addressing upcoming health care challenges.

In the past decade, a crucial role for commensal gut microbiota in health and disease has been identified, putting this research at the epicentre of modern medicine. The dynamic nature of the gut microbiota in early life is well known, but more subtle changes in diversity continue until middle age (approximately 40 years in humans) at which point there is a period of relative stability followed by age- related changes later in life. It has been suggested that the age-related changes in microbial composition are a form of ‘dysbiosis’ that contribute to host immune remodelling and age-related diseases. Yet the point at which the stability in the microbiome is lost and age-related changes in health start, remains poorly understood. Furthermore, the pathways by which microbiota exert their effects on immune remodelling and health conditions remain elusive. This work will address these knowledge gaps and in doing so, provide new insights into microbial-driven mechanisms of immune dysregulation with age.

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

The major goal of this project is obtain an understanding of how microbiota shape the microenvironment and immune landscape to affect healthy ageing.

Our primary output of this project will be the generation of vital data and knowledge to help understand features of the microbiota that change with age, sex and frailty. This will allow us to identify potential diagnostic and prognostic microbial biomarkers of early immune decline and explore potential intervention strategies (e.g. metabolite depletion/rescue studies). Such outputs will be disseminated through publication in open access, peer-reviewed journals and presentations at conferences, seminars and workshops. Appropriate data outputs including methods (e.g. large datasets) will also be published in open-access repositories. The lab will actively participate in public engagement events to increase public awareness of our work, its implications for health and disease and importance of biological research. The findings from this research will allow us to investigate novel therapeutic interventions that restore immune function and/or hematopoiesis with age, and perhaps, ultimately provide the healthy old age that we all aspire to.

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

The principal end-users of this research include older adults who are frail and are at increased risk of age-related health conditions such as infectious disease, cognitive decline and cancer. Our long-term goal is to find a beneficial and effective therapeutic strategy to reduce morbidity and improve the health-span of this population.

In the shorter term (over the first 5 years of the project), the primary beneficiary of this work will be the academic community. This work will create projects for trainees as well as the opportunity to build new research relationships with other academic fields. The published findings from these studies will attract future funding, which will benefit the research institution & researchers.

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

As this work is interdisciplinary, we will communicate findings by publishing in journals and presenting at local, national and international conferences and seminars that reach across a broad spectrum of biological and medical disciplines. To ensure maximum dissemination we will only publish in journals with available open access policies. Furthermore, to speed up access of data and knowledge, findings will be published on open access preprint servers such as BioRxiv. We will also aim to publish all large data sets generated (including negative results) and methods used for analysis on repositories such as Github or OSF (open science frameworks), allowing researchers to rapidly access and utilise data and avoid unnecessary repetition of experiments. To enable rapid translation of findings to the clinic, we will exploit new and existing collaborators and utilise local clinicians for access to clinical samples. In this respect, the local environment within the Institute of Medical Sciences linked to the NHS Grampian Biorepository is ideal for developing collaborations and translating findings from animal models to a clinical setting.

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.

The point at which age-related changes in health start and the mechanisms driving these changes remain poorly understood. As such, our procedures span the life stages including young (<3 months), mature adult (3-6 months), middle-aged (10-14 months), and old (18-28 months) mice. Even though mice are genetically identical (C57Bl/6 background) and have the same diet and the same environment, they demonstrate significant variability in weight, frailty, metabolism and strength, recapitulating the human condition. Some mice age well (i.e. live to two years and are not frail) and some of them age poorly (i.e. die early and have a significant or protracted period of frailty). Like humans, mice experience social stressors (i.e. dominance and submission), range from underweight (<5%) to overweight (>20%) and make very different choices about voluntary exercise, with some mice using the exercise disc extensively and others not at all. This heterogeneity provides a unique opportunity to study non-genetic associations with healthy/unhealthy ageing and assess the interaction among the gut microbiota, the immune system, epigenetics (which controls which genes are turned "on" or "off"), intestinal pathology and frailty. The longitudinal nature of this work will allow us to address questions such as whether changes in the microbiota proceed and predict immune remodelling or vice versa. Answering these

questions will direct future therapeutic strategies with the goal of improving the health-span of ageing individuals.

In this Project we will use "standard" specific pathogen free (SPF) C57Bl/6 wildtype (WT) mice and genetically altered mice, such as TNF-α knockout (TNF-/-) mice on a C57Bl/6 background. With age, there is an increase in inflammation that can be measured by tumour necrosis factor-α [TNF] in the blood. Individuals with higher than average levels of inflammation (i.e., TNF) are more likely to develop chronic health conditions or frailty, be less active, and face premature mortality. Unlike the WT mice used in this project, the genetically altered mice do not get age-associated inflammation, and as such, they age better. We compare WT and genetically altered mice (e.g. TNF-/-) to understand which effects of age, such as changes in the gut microbiota (bacteria, viruses, fungi etc), changes in the immune system, changes in tissue structure etc. are inflammation dependent.

With age, there are also changes in epigenetic regulators that dictate which genes are turned "on" or "off". Changes in epigenetic regulators can influence hematopoeisis, which is how immune cells are made. Consequently, age-related changes in epigenetic regulators have a significant impact on health conditions including cancer, cardiovascular disease and stroke. In this Project, we use knockout mice that are missing certain epigenetic regulators, such as ten-eleven translocation-2 (TET2) knockout mice (Tet2-/-). TET2 mutations increase exponentially in humans with age, occurring in at least 10% of older adults. We have recently shown that targeting age-associated inflammation may have protective benefits in individuals with aberrant epigenetic regulators. The mice used in these experiments will not be aged past 14 months, as we have previously found that a large proportion of the Tet2-/- mice develop tumours at approximately 15 months. We feel that this adds too much variability and uncertainty to the data, resulting in poor experimental design, ethical and welfare considerations (re: 3Rs). As such, we have elected to use slightly younger mice (<14 month) who are much less likely to have developed tumours (~7%).

For all experiments, both male and female mice will be used to capture heterogeneity in the ageing trajectory. SPF animals are used as several infectious agents have been identified over the years to have either adverse effects on animal health and/or research outcomes and decreases lifespan.

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

In Protocol 1 we will breed and maintain GAA mice. These mice will then be used in Protocol 2.

Typically, mice in Protocol 2 will be raised into old age, or used as young controls for age comparisons. We will collect blood and fecal samples longitudinally from all mice beginning at 3 months of age to analyze the gut microbes, immune and metabolic parameters as well as epigenetic changes. A maximum total blood volume of <15% will be taken within a 28 day period and a maximum of 10 bleeds will occur in any mouse over their life-course (inclusive of all procedures). For each bleed, we will use the most refined approach to acquire the necessary volume as per the NC3Rs recommendation. In addition to these biological measures of ageing, we will assess behavioral changes that occur with frailty including changes in muscle strength, mobility and cognitive behavior.

These measures are non-invasive and may each be performed multiple times (usually in early and late life, and pre/post intervention). These measures will help us characterize

features that change with age, sex, and frailty (i.e. healthy vs. unhealthy ageing trajectories).

We will then see if we can influence the ageing trajectory. For this, we may do any one of the following:

i) switch cage bottoms to transfer microbiota from one group to another (e.g. old mice to young mice) or co-house young and old mice. From past experiments, we have not had any increase in the instances of fighting by doing this. ii) change the mouse diet (e.g. to a high-fat diet), or iii) administer a potential therapeutic. This would occur using using an intraperitoneal injection (i.e. injection into the lower part of the abdomen) or subcutaneous injection (i.e. injection between the skin and muscle). The mice will receive 3 back-to-back doses of antibody or isotype control (e.g. day 1, 2, 3), and receive additional doses every 2-4 days for 2 wks maximum (most experiments are 2 wks to maintain depletion.

In many cases, we will also measure differences in intestinal barrier permeability with age using a fluorescent dye called FITC-dextran. Briefly, mice will be fasted 0-6 hours prior to administration of 150uL (80 mg/mL) FITC-dextran using a gavage needle. Blood (<150 uL total) will be collected 0, 1, 2, and 4 hours later using a needle puncture to the tail vein and the amount of fluorescent dye to enter circulation will be calculated.

In some experiments, mice may be given Streptococcus pneumoniae to induce pneumonia, one of the leading causes of morbidity and mortality in older adults. The mice will receive one dose of pathogen through the nose following brief exposure to inhalation anaesthetics. Experiments will look at the immediate immune responses in the first few days, but may also last as long as 12 weeks post- infection to allow for assessment of post-pneumonia sequelae. In some experiments, mice may be administered a substance that modulates inflammation or epigenetics prior to or during infection as a potential therapeutic to improve infection outcomes.

Overall, the cumulative experience of mice will typically be expose to between 2 to 3 experimental procedures (if repeat bleeds or dosing are regarded as a single procedure) that may each cause short but usually separate periods of typically mild or potentially moderate degrees of suffering.

Overall number of bleeds: A maximum total blood volume of <15% will be taken within a 28 day period and a maximum of 10 separate bleeds will occur in any mouse over their life-course (inclusive of all procedures). Bleeds will be performed using the most refined method as per NC3Rs for the required volume.

Overall number of injections: A maximum of 20 injections will occur over the course of an animals life. This includes all injection routes.

Experiments will end with animals being humanely killed. For the majority of experiments, this will involve euthanizing the animal under terminal anesthesia using exsanguination (draining of blood) followed by cervical dislocation. This method is used to collect a large volume of blood for analyses. In some instances (approximately 10%) we cannot use cervical dislocation as it may cause contamination of the bone marrow immune cells (e.g. when studying haematopoiesis, which is how immune/blood cells are made). In these instances the animal will be euthanize under terminal anaesthesia using exsanguination followed by cutting of a major artery to ensure cessation of circulation.

Exsanguination/fixation perfusion (i.e. the administration of a chemical substance into the vascular system in order to distribute fixatives throughout the tissues) under general anesthesia will be used when tissues are required for histology.

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

The vast majority of WT and TNF-/- mice will experience no adverse effects or only mild adverse effects, from birth to 18 months of age. Our protocols for colony care and maintenance of an ageing mouse colony results in better health than reported by the National Institute of Aging (NIA)'s ageing mouse colony. Once mice reach old age (>18 months) they are similarly expected to range from no adverse effects to mild adverse effects; however, mice experiencing mild distress may cumulatively be categorized as moderate due to the longevity of the project. Old mice often exhibit clinical signs that would indicate disease in younger mice but are considered part of the normal progressive decline in health that occurs with aging. A few signs that are considered normal in an older mouse include decreased body condition, increased respiratory rate and pallor. In addition, due to their background C57Bl/6 mice are predisposed to age-related hearing loss, alopecia and dermatitis, barbering, skin lesions, idiopathic ulcerative dermatitis, ocular lesions, ocular ulcers, cataracts, palpable masses (tumours), and rectal prolapse. While these are considered part of the normal progressive decline in health that occurs with ageing in C57Bl/6 mice, they can decrease the quality of life. Animals will be monitored closely and when reaching the endpoints will be humanely killed. The veterinary staff will also be consulted on any potential treatments.

Similarly, the Tet2-mutant mice are expected to have no adverse effects or only mild adverse effects from birth to 14 months of age.

Our pneumonia model is expected to have predominantly mild but may have some moderate effects. For instance, mice may be hunched or have abnormal breathing during peak infection. To reduce the adverse effects on the animals, we provide mice with supportive care during infection (e.g., hydration, warmth and extra nutrition). In our experience, this significantly reduces the number of animals that reach endpoint (70% of our older mice survive). Old mice that have fully recovered from infection (>14 days) may demonstrate reduced mobility and learning defects compared to young mice or old mice that were not infected, yet they have no indication of pain and distress and are well groomed, alert and in normal body condition. This post-pneumonia sequelae, or slow-progressive decline in health following pneumonia, is one of the phenomena our laboratory studies.

In all experiments, animals will be carefully monitored and humanely killed before they exceed moderate severity limits. Guidance will be sought from the NVS and 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)?

We expect approximately 30% of mice to experience mild severity and 70% to experience moderate severity, after accounting for all potential procedures and cumulative effects across the life-course.

What will happen to animals used in 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?

Ageing is an extremely complex biological process that results in a progressive decline in function across multiple organ systems. The mammalian immune system alone requires co-ordinated interactions between many different cell types and molecules, including input from the circulatory and lymphatic systems. Immune remodelling with age could occur due to changes in bone marrow haematopoiesis, or alternatively be due to issues with thymic homing or cell maturation. Similarly, it is believed that gut inflammation promotes dysbiosis because it alters local redox and thus specific nutrients/growth factors which gives some microbes a competitive advantage. However, loss of tolerance in the gut during ageing may proceed inflammatory responses. In vivo systems are therefore required to shed light on the key mechanistic cell-cell and cell-microenvironment interactions that influence tissue inflammation, physiology and pathology with age and in response to pulmonary challenges. Unfortunately, in vitro assays currently cannot fully emulate the complexity of the biology of ageing. As such, in vivo models are essential to get an accurate picture of how whole-body processes change dynamically and contribute to unhealthy ageing and frailty.

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

- In vitro cell culture assays
- Organoids, organ-on-a-chip
- Human tissue and data

Why were they not suitable?

For decades, most research on the biology of aging has focused on an independent mechanistic understanding of the genes, pathways, molecules, and cells that change with age and that may improve or worsen aging processes. Although this has yielded excellent insights, it has failed to produce a thorough mechanistic understanding of how these components of biological systems interact in time and space and change with unhealthy ageing (i.e. frailty). As a result, research needs to explore how multiple mechanisms interact across different spatial and temporal scales to promote healthy ageing. In some cases, non-animal alternatives are suitable and have reduced the number of animals required in this project. For instance, to investigate the impact of certain inflammatory mediators (e.g. TNF, which contributes to age-associated inflammation) and microbial metabolites on intestinal permeability, we will measure the electrical resistance of a barrier pre- and post-treatment using cultured cells. Identifying which microbial products/metabolites and immune factors change with frailty and age, however, first requires an in vivo model. In addition, even the ability to co-culture several different cell types together to understand cell-cell interactions is not reflective how immune-gut interactions works, nor does it take into account gut physiological changes with age. While

we considered using human tissue, it is hard to imagine a situation in which frail older adults would be recruited to donate gut biopsies in the numbers required to disentangle the effect of sex, medications, health conditions, frailty and age. The shortened lifespan of mice allows us to collect samples longitudinally across the life-course, which wouldn't be feasible in any individual human. With respect to immune dysfunction with age, a lot of immune and tissue resident cells isolated from a tissue out of their micro-environment and put into artificial culture vessels will change their physical and physiological phenotype and functions and are therefore not accurately representative of the human condition.

More sophisticated culture techniques such as human 3D lung or intestinal slices can be used whereby tissue resident cells including resident immune cells can be studied 3D within the natural lung / gut architecture. Whilst tissue slice cultures represent a significant advantage over traditional in vitro assays, they still lack input from the circulatory system and therefore cannot be used to study cell recruitment. Organoid cultures are another technique that would allow us to study some limited aspects of cell-cell interaction and function but they cannot replicate tissue and cell migration changes that occur with biological ageing.

The experiments that require dynamic tracking of cells and how they function in vivo is not possible with human tissue biopsies. Additionally, mechanistic studies require manipulation of the tissue environment or cells in vivo and this cannot be done experimentally in humans.

Overall, none of the alternatives investigated will fully replicate the lung/gut tissue environment and the way in which circulating immune cells interact with resident cells. However, we will regularly investigate alternatives throughout the duration of the project from resources such as NC3Rs and FRAME to ensure that any suitable techniques and advances to replace animals are put in place in our programme of work. In addition, we will utilize human samples to compliment our animal models wherever possible. For example, we will be investigating age-related changes in microbiota using human stool samples and will use these samples to investigate changes in barrier function in vitro, using trans-epithelial electrical resistance (TEER) experiments. Overall, alternative techniques will be used throughout the project to guide, complement and translate our in vivo experiments, thereby reducing the numbers of animals required for in vivo 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?

Numbers of mice are based on previous experience using these experiment techniques during my career and NC3Rs recommendations on effective breeding and maintenance of transgeneic mice. Numbers take into account breeding strategies for genetically altered mice and the numbers of animals we anticipate using in the planned experiments across the course of this project.

In line with previous activity for the lab, we expect to use 5000 transgenic mice which takes into account important littermate breeding strategies for experiments and maintenance of transgenic colonies; with an estimated 3000 mice transferred to

experimental protocols. Additionally, we anticipate using 3000 wild-type mice in experimental protocols.

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

We used the Experimental Design Assistant (EDA) on the NC3R's website. Any new staff members or students joining the group will be trained in using the EDA and will be encouraged to use it throughout the course of their individual projects and update their training with online courses offered by NC3Rs.

The EDA allows us to review and discuss practical steps (e.g. randomisation, blinding) and data analysis (e.g. sample size and appropriate analysis tests). Additionally, all new lab members are trained in statistical analysis methods to ensure statistical significance with minimum number of mice, and consultation with statisticians whenever necessary. Plans for experiments, including approaches for data analysis will be discussed routinely in lab meetings so there is agreement that best practices and methods are being followed.

For all experiments we use inbred, SPF mice to reduce inter-animal and environmental variability. Most experiments also utilise littermate animals for both wild-type and genetically altered mice to reduce cage effects on the microbiota. Other variables such as age and weight are also taken into consideration for all studies. Overall, these strategies limit variations allow us to reduce the numbers of control animals/groups required in experiments. We also routinely use both sexes (control matched) allowing for a larger percentage of mice produced during breeding to be used in experimental protocols.

Finally, we have refined our techniques to enable multiple parameters to be measured from mice across time and space (e.g. one colon is routinely divided to provide cells for phenotyping, tissue for RNA, tissue for histology and imaging) which reduces the number of animals required to answer our objectives.

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

Breeding of genetically altered animals is a large portion of our overall animal use throughout the project. We use intermittent breeding to stagger and control the number of mice at each life stage. This allows for sufficient time between planned experiments using aged mice. When mouse lines are not being used experimentally, breeding goes onto a tick-over protocol to ensure only the minimum number of animals are bred to maintain the line as per NC3R guidelines.

Our littermate breeding strategy also allows for littermate animals to be used as controls in the majority of experiments reducing the numbers of out-sourced wild-type mice required. Discussions about breeding lines and numbers of animals will be discussed monthly in lab meetings to make sure we accurately plan for future experiments and do not overbreed any mouse lines. Importantly, we work closely with experienced animal facility technicians to optimize breeding strategies to reduce animal numbers.

During our routine meetings with the lab and other Immunology groups, we will discuss all animal experiments from design through to analysis and results. Such discussions ensure all animals are used in effectively and allow for experiments to be combined or tissue shared across multiple researchers. This is an important strategy to reduce animal usage. In addition, we routinely harvest and fix or freeze as many relevant tissues as possible post-mortem for later use. This approach has been used effectively to assess immune

responses in other organs (e.g. pleural cavity, liver, heart) and has highlighted potential avenues for analysing inter-tissue communication across the life-course.

Pilot experiments are routinely used to determine the appropriate numbers of animals required to achieve statistical power and to ensure feasibility. To ensure reproducibility and reveal less pronounced

effects, experiments are performed on a minimum of two separate occasions and data pooled for statistical 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.

This project will use mouse models to study how age-related changes in immune cells, the gut microbiome, epigenetics and the ageing microenvironment improve or worsen the aging process, resulting in frailty. Old WT C57Bl/6 mice are the most appropriate model to use in this in vivo setting as the immune system, tissue organization, and the ageing microenvironment of these mice are similar to humans, resulting in similar age-related pathologies (e.g., frailty). Ageing these mice is the most refined method to examine the underpinning mechanisms of frailty onset and progression.

TNF-/- mice are also used in this project. These mice do not develop age-associated inflammation and as a result, age better than their WT counterparts. We use these mice to disentangle the impact of the ageing inflammatory microenvironment from other age-related changes (e.g. in gut physiology).

The genetically altered mice used as a model of epigenetic changes (e.g. Tet2-/- , Tet2-/+ and Tet2f/f) are prone to developing tumours with advanced age. As such, we have refined our methods to only include these mice up until 14 months of age. This ensures that the animal is not in discomfort, and has significantly reduced heterogeneity in our data from the tumours.

The following methods are non-invasive and should not cause pain, suffering, distress or lasting harm: frailty assessment, cognitive assessment, microbiota colonization, dietary interventions.

Blood samples will be collected from mice using the most refined method for the required volume and allowing for sufficient time for the animal to recover between blood sampling sessions, as per the NC3Rs guidelines. A maximum total blood volume of <15% will be taken within a 28 day period and a maximum of 10 separate bleeds will occur in any mouse over their life-course to account for cumulative suffering.

Our FITC-dextran method is the least invasive, non-terminal, method to measure intestinal permeability. Similarly, we use the most refined tolerance test approach to assess metabolic function.

Mice that are used in our model of pneumonia are anesthetized using isoflurane as an inhalant anaesthetic to reduce stress during intranasal administration of the bacteria. Once infected, we provide supportive care (e.g., hydration, warmth and extra nutrition), to reduce the amount of pain, suffering and distress and check the mice regularly (2x daily during peak infection) to ensure that if a mouse is nearing endpoint, it is identified and humanely killed. This inhalant model is less invasive than alternative pneumonia models (e.g., intratracheal models).

To assess leukocyte migration, mice will be injected intraperitoneally with a chemokine that signals leukocyte migration to the peritoneum. Small injection volumes will be used to minimize discomfort and mice will be euthanized within 14 hours to ensure no lasting harm.

Immune cell/cytokine depletion experiments will be done using IP injections and small injection volumes of non-irritant and near-neutral pH antibodies. This should cause minimal pain or discomfort. Some immune cells (e.g., monocytes and macrophages) will be depleted using microparticles that require an intravenous injection. For this, we use the tail vein which causes only minimal or transient pain and distress.

We will be administering a drug called 5-azacytidine as a potential (beneficial) therapeutic intervention. Small volumes will be administered IP once daily for 5 consecutive days, which is necessary to impact hematopoiesis and immune responses.

We will continue to assess our methods, consult with collaborators and review the literature to continually refine our approaches so as to cause minimal pain, suffering and distress while upholding the best possible practices and scientific approaches.

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

To our knowledge, there are no other species of lesser sentience that can fulfil the requirements of this project to the same extent as the laboratory mouse. We are investigating complex and fluid processes in relation to biological ageing, including but not limited to age-related changes in immunity, host- microbe interactions in the ageing gut, and changes in epigenetics in the periphery and bone marrow environment. Less sentient animals such as drosophila do not possess acquired/adaptive immunity and have a very low diversity gut microbiome (~ 5 species). As such it would be difficult to acquire meaningful results surrounding immune-gut-epigenetic interactions.

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

There are unique welfare issues that need to be addressed when maintaining ageing mouse colonies including age-related decline, debilitation, frailty and pain associated with chronic diseases. It is vital that special care be taken in the maintenance of these colonies as well as during experimental design and implantation. Preventing premature death requires that all age-related deficits be minimized in colonies of ageing mice, including but not limited to stress, age-related changes in the immune system, chronic conditions, various cancers and other late-life diseases.

To maintain health and life quality, it is essential for aging mice to remain physically and mentally active. To achieve this, our mice are put on a specialized ageing diets as we have found that feeding diets that restrict calories increases maximum longevity, reduces the incidence, and delays the onset of several cancers and late life diseases as well as retards the biological changes that occur along with ageing. We have found that long-term

exposure to environmental enrichment and refined handing methods also stand out as very useful strategies to extend longevity. Mice that exhibit heightened reactivity to stressors and chronic anxiety have accelerated immune senescence and tend to die prematurely. Refined handling methods and extra environmental enrichment is effective in significantly reducing these stress responses by improving innate immunity. This may result in lower susceptibility and vulnerability to bacterial and viral infections during old age. Alongside extra enrichment and specialized diets, aged mice are given an exercise disc, which we have found to reduce age- associated obesity and improve overall mobility in aged mice.

Old mice (18-28 mo) have a thorough health check done biweekly to ensure that we identify any pain, suffering or distress with increasing age. Each mouse is examined for body condition, malocclusion, dehydration, eye ulcers or cataracts, tumours or lesions and any changes in respiration rate. In addition, we frequently score old mice using a clinical frailty index for mice. This in-depth assessment of health scores >30 different parameters of unhealthy ageing. Any mouse with increasing frailty scores is monitored closely until endpoint. We know from experience that approximately 1/3 of the wildtype mice will develop progressive frailty starting at about 18 months of age, about 1/3 will develop a score of 0.5-1.5 that will remain stable until the end of life and the remaining 1/3 will have a precipitous increase in frailty at the end of life (generally 18-24 mo). The TNF-/- experience less frailty than the wildtype mice, but will be similarly assessed.

Procedures will be performed by trained and skilled personal license holders, who will handle animals with care. All animals are monitored for adverse effects using objective measurements of clinical signs associated with adverse effects. Increased monitoring will occur if animals show adverse effects prior to humane endpoints being reached.

We will continue to use the latest techniques in animal handling (e.g. tunnel handling) in order reduce the stress associated with any procedures on mice. Where ever possible, the least invasive methods for dosing and sampling will be used throughout the project. Single-use needles will be used for all blood sampling / injections, and the number of attempts to take a blood sample will be minimised (no more than three needle sticks in any one attempt), as per the NC3Rs guidelines. For some procedures, we use anaesthesia where appropriate and suitable (e.g. for humane restraint during intranasal administration of substances). Such procedures will be discussed with the NVS and experienced animal technicians to ensure they continue to be the most appropriate technique.

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

We follow the LASA guidelines (https://www.lasa.co.uk/current_publications) and ARRIVE guidelines (https://arriveguidelines.org/) for best practice and follow PREPARE guidelines (https://norecopa.no/prepare) for experimental planning. Additionally, we routinely consult NC3R’s resource library (https://www.nc3rs.org.uk/3rs-resources) including online training courses and videos for maintaining and improving best practices. The lab uses the NC3Rs Experimental Design Assistant to ensure all experiments are designed in a way that will allow us to achieve statistically significant results with the minimum number of animals required.

The lab will also routinely discuss 3Rs during lab meetings to ensure everyone actively considers all aspects of the 3Rs. Additionally, we read, share and discuss publications from other groups doing similar experiments to ensure the most refined procedures are being used for the topic being investigated. Collaborators and other researchers working on similar models will also be consulted on a regular basis.

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

We will hold frequent discussions with NACWOs, NIO and NTCO and dedicated veterinarians to seek guidance to make sure we maintain the highest level of animal welfare and constantly refine our animal use. We also will continue to present our research and studies to staff and get feedback from expert technicians to ensure we are following current best practices.

The lab will keep track of the latest advances in improving animal welfare via discussions with colleagues, attending conferences and assessing published literature. We routinely consult the NC3Rs resources page and FRAME website to ensure we are informed about all the latest advances in 3Rs and new approaches to using alternatives for animal research. The lab will also attend any appropriate online seminars and training offered by NC3Rs or in house.

68. Neuroplasticity mechanisms underlying sensory perception, learning and memory

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

Perceptual processing, Brain plasticity, Brain circuitry, Neuroregulation

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

The principle aim of this project is to understand how the brain adapts to new information with a focus on sensory, perceptual and higher forms of learning and memory.

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 undertaken during this project will lead to enhanced understanding of the brain regions, connections between brain regions and neurotransmitter systems involved in perceptual processing and learning-related plasticity [adaptability to changes in environment and experience].

The brains of rodents, although not as developed, share similar structure and function to human brains allowing for appropriate translation with the human studies being undertaken

as part of this research. A value of using mice and rats, is the ability to separate out different behaviours and link with specific brain circuitry, neurotransmitters and genes that are conserved between rodents and humans. Much successful work has previously been undertaken using rodents, including pre-clinical testing of numerous drugs and therapies leading to treatment of human disease. By using rodents rather than non-human primates for this work, we are arguably using less salient animals in keeping with the principals of the 3Rs.

Magnetic resonance imaging (MRI) is an excellent technique to use to further understand the brain regions important during these processes as it can give a global measure of whole brain activity. For example by imaging the brain before, during and at time points following training we will be able to discern whole brain anatomical changes. We will also test new preclinical drugs that for example enhance or inhibit neurotransmitter signalling.

The work undertaken in this project is informed by a transdiagnostic approach [an approach that focuses on common underlying mechanisms that present across numerous brain disorders]. The project will lead to greater insight into the contribution of prior knowledge and primary sensory processes on learning and memory which are known to be altered in neurodevelopmental and stress- related disorders such as schizophrenia, autism, depression and post-traumatic stress disorder (PTSD). The work undertaken will therefore potentially lead to new pharmacological or behavioural treatments for these conditions. Mental health disorders afflict around 17% of adults in the UK population and autism and schizophrenia are estimated to have a prevalence of around 2%.

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

Outputs from this project will include scientific publications on the neurochemical changes underpinning global brain structure and function as well as the specific brain circuitry important during perception, learning and memory. Conference communications including invited plenary lectures, symposia talks and posters, as well as public engagement activities will also be undertaken.

As a translational research project, this research aims to inform the development of new pharmacotherapies for a range of common but debilitating brain disorders in humans.

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

The work undertaken in this project investigates perceptual processing deficits which are fundamental to many brain conditions including autism, schizophrenia, depression and PTSD. Autism and schizophrenia are estimated to affect 1.3 million people in the UK, around 2% of the population. It is also estimated that around one-fifth to one-half of schizophrenic patients are treatment resistant.

Mental health disorders afflict around 17% of adults in the UK with approximately one-half of patients with generalised anxiety disorders failing to respond to initial antidepressant treatments. The scientific community will benefit from the advance in mechanistic understanding of perceptual and other forms of learning. The identification of drugs that rescue behavioural deficits may lead to the development of new treatments.

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

The findings of this research will be communicated at national and international scientific meetings, published as preprints (e.g., biorxiv), and published ultimately in peer-review journals. Data will be made available wherever possible in open access repositories. This will ensure our research is available to other researchers and the general public, including null findings. We will also collaborate with other research groups to ensure techniques, expertise and any unsuccessful approaches are shared and refined.

Species and numbers of animals expected to be used

- Mice: 4060
- Rats: 1160

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 aim to understand the neural circuitry and connections between brain regions during perception, learning and memory. We therefore need to test intact behaving animals to make this work scientifically relevant. Rats and mice are widely used as the mammalian species of choice for investigations of the central nervous system. They are particularly favoured for selective gene targeting experiments and can be trained on complex touch screen tasks that are readily translatable to humans. We will primarily be using adult animals, however, for a limited number of experiments we will need to undertake maternal separation of pups to be able to look at the impact of early life stress on brain development and behaviour in later life.

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

Animals will be bred in-house or purchased from a commercial breeder and habituated to the animal facility for at least seven days before procedures commence. Animals will be socially housed with appropriate levels of enrichment (e.g. chew blocks, and nesting material). Some animals will undergo food restriction with body weights being monitored at least three days per week to ensure their body weights do not drop below 85% of their maximum individual weight. Animals will then be trained on complex behavioural tasks reinforced with a food reward. Some animals will undergo behavioural testing with head-fixation. They will be gradually habituated to the head-fixation environment and trained for a maximum of 2 hours per session, with a maximum of one behavioural session per day. It may also be necessary to administer miotic eye drops to some animals, to constrict the pupil, enabling eye tracking in low light settings.

Some animals will undergo stereotaxic surgery [surgery that uses a three-dimensional coordinate system to locate brain regions of interest] to implant devices needed for subsequent experiments such as head-fixation brackets for some behavioural testing, electrodes to undertake electrophysiology [a technique that enables the recording of electrical activity in biological tissue] or drug delivery probes for infusion of drugs directly into a brain region of interest. If animals are damaging the shielding/implants of their cage mates, or extensively fighting they may be single housed as a last resort.

Some animals will undergo anesthetised magnetic resonance imaging (MRI) sessions to measure changes in brain structure for a maximum of 4 hours per session. Anesthetised MRI will enable structural changes to be compared between testing sessions for example

to assess differences between short and longer memory time points. Other animals will undergo awake MRI sessions to measure changes in brain function to specific stimuli or events. For awake MRI sessions animals will be restrained in the MRI scanner using head-fixation brackets previously implanted during surgery.

They will be gradually habituated to the scanner environment and scanned for a maximum of 2 hours per session. Awake MRI sessions will enable changes in brain activity to be measured during active perception, learning and memory recall.

Some animals will be administered substances known to modulate neuron [nerve cell], or glia [non- neuronal cell] function. These could be administered via a number of routes for example intraperitoneal, subcutaneous, or intracerebral via cannulae previously implanted during surgery.

Following undertaking procedures all animals will be humanely killed.

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

Reductions in body temperature can occur under anaesthesia during MRI or surgery. We expect animals undergoing surgery to develop clinical signs such as redness and scabbing around the implant site, but that these animals will make a rapid recovery post-surgery. It is essential for our experiments that animals are in good health in order to be able to perform behavioural tasks and undergo imaging.

However, on rare occasions following surgery the wound may become infected, or re-open and cause discomfort. The brackets for head-fixing, electrode implant or cannulae may come loose and cause discomfort. Implantation may cause unintentional damage to sensory or motor regions of the brain leading to adverse neurological signs such as sensory loss or motor deficits. In the case of infection, if appropriate, antibiotics will be administered. In the case of re-opening, in the absence of infection, the animal will be re-anaesthetised, and the wound may be closed if appropriate (once only). If an implant becomes loose or broken, the animal may be re-anaesthetised, and the implant repaired or re-fastened if appropriate (once only). If we observe any abnormalities in motor behaviour after surgery such as impaired walking on one side of the body the animal will be humanely killed. Analgesia will be used post-surgery to alleviate pain, suffering, distress or lasting harm.

We will administer substances that are expected to modulate brain function. Expected effects of these may include mild changes in performance on behavioural tasks and subtle changes in brain activity measured for example using electrophysiology and/or MRI. Other side effects may occur with these substances, such as impaired coordination, changes in social behaviour, hyperactivity, and/or increases in anxiety.

Head-fixation can be stressful to ameliorate this, animals will be gradually acclimatised to the head- fixation environment over a number of days. Animals are typically trained in a virtual reality set-up allowing for self-initiated training by the animal running.

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 10%,
Moderate 90% Rat
Mild 10%,
Moderate 90%

What will happen to animals used in 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 understand the neural circuitry and connections between cortical layers and other brain regions during active perception. We therefore need to test intact behaving animals to make this work scientifically relevant.

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

The research undertaken in this project will be complemented by work undertaken using human subjects. We also considered using organoids [organs produced in-vitro], in-vitro [outside of a living organism] and in-silico [computer based] approaches.

Why were they not suitable?

The direct study of neural circuits in humans is not possible as MRI is an indirect measure of neuronal activity. We therefore need to combine it with other invasive methods such as calcium imaging [technique to measure calcium signalling] and optogenetics [technique to control cell activity using light] to gain an understanding of mechanisms at the integrated cellular level. We also aim to understand the role of neurotransmitters such as Gamma-aminobutyric acid (GABA) during perception. To do this we need to undertake pre-clinical pharmacological studies [studies investigating the properties and reactions of drugs]. If the drugs we test show promise they will potentially be tested in human subjects. Organoids, in-vitro and in-silico approaches are currently not available as a realistic alternative to achieve our objectives of understanding the integrated complexity of cortical [outer layer of the brain] and subcortical [deeper brain regions] microcircuits.

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 previous published literature and data from our collaborators to estimate our group sizes to ensure the minimal number of animals are used while maintaining valid statistical conclusions. Where we use between-subjects [e.g. participants are either in a

treatment group or a control group] or mixed designs [incorporating both between and within-subject measures], we ensure that the control groups have as similar an experience as possible to the experimental group, besides the key manipulation of interest. Where we use within-subjects [e.g. participants are exposed to all treatments] to capitalise on individual variability in behaviour, we ensure that we counterbalance the order of drug doses so that this factor can be considered in our statistical models. Experiments will be blinded and randomised wherever possible.

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

Our experiments involve longitudinal experiments undertaking behavioural testing, pharmacology and brain imaging to assess activity at different time points within the same animal. This enables within- animal comparisons and reduces the overall number of animals needed. MRI is typically used as global measure of brain activity therefore giving us an understanding of relevant brain areas to probe with more invasive methods leading to more targeted experiments. Our MRI methods are directly comparable with human studies enabling the potential for considerable clinical benefit. We consult the NC3Rs' experimental design guidance and experimental design assistant (EDA) to support 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?

Animal breeding will be undertaken in an efficient way to ensure more animals are not produced than is needed for experiments and the maintenance of colonies. We will also consult the NC3Rs breeding and colony management resource.

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 aims to understand the neural circuitry and connections between brain regions during perception, learning and memory. We therefore need to test intact behaving animals to make this work scientifically relevant. Rats and mice are good model animals given much work has previously been undertaken using them, therefore the scientific literature can be consulted on basic aspects of the work

e.g. to ensure appropriate drug dosing. The brains of mice and rats, although not as developed, share similar structure and function to human brains allowing for appropriate translation with the human studies being undertaken as part of this research. MRI is a non-invasive procedure and the behavioural tasks we will use do not cause any pain, suffering, distress, or lasting harm. Some of the animals will be required to undergo surgery and anaesthesia, however, analgesia and careful monitoring will be used to alleviate pain, suffering, distress or lasting harm.

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

The brains of mice and rats share similar structure and function to human brains allowing for appropriate translation. Other species such as octopus or fruit flies have quite different brain anatomy and are therefore not as suitable for translation to human studies. Although other species such as octopus and fruit flies have traditionally been classed as less sentient, it is highly contentious whether they share the same neural architecture as rodents and humans for the types of learning underpinning sensory and perceptual representations of relevance to behavioural and developmental disorders of the mammalian nervous system.

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

The animals will be given cage enrichment such as running wheels and will be housed with their littermates wherever possible. They will also be kept on a reverse light-dark cycle wherever possible resulting in less disturbance to their natural wake/sleep patterns during experimental testing. We will ensure that all experimenters working under this project licence are well trained in animal handling to avoid unnecessary distress or anxiety. Pilot studies will be performed to ensure continued refinement for example to adjust doses of pharmacological agents prior to testing additional mice. Analgesia given following surgical interventions will also be reviewed regularly. We will continue to update our procedures to improve the welfare of our animals. Ensuring our animals are healthy is of the upmost importance to ensure robust and valid experimental outcomes.

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

PREPARE and ARRIVE guidelines will be used to assist with experimental planning and evaluation. The NC3Rs resource on refining head fixation and fluid control in rodents will also be consulted.

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

Information on the NC3Rs website will be regularly checked. We are signed up for the NC3Rs newsletter and attend regional 3Rs symposia. The NACWO and NVS will also be consulted along with The Laboratory Animal Science Association (LASA).

69. Mechanisms of Acute and Chronic Kidney Injury

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Kidney Injury, Transplant, Acute Kidney Injury, Renal Fibrosis

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 main aims of the project are (1) To understand 'kidney injury' and (2) identify new ways of treating 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?

Kidney Injury is very common and occurs in many clinical situations such as sepsis, heart disease, and after kidney transplantation. The main cause of kidney injury in these scenarios is 'Ischaemia Reperfusion Injury', when blood supply (and oxygen) to kidneys is interrupted for a period of time (ischaemia - when blood supply (and oxygen) is not being delivered; reperfusion - when blood supply (and oxygen) is reinstated). To date there are no specific treatments for it. Understanding what happens in kidney injury at the 'molecular' and 'cellular' level and finding new novel ways of treating it will transform lives in a variety of clinical situations.

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

We expect to gain a better understanding of how kidney injury occurs and test novel treatments that can reduce it. These findings will lead to publications and potential development of novel therapies/drugs.

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

The main goal of this project is focsued on understanding the key molecular and cellular mechanisms of kidney injury (acute and chronic) and identifying novel methods of manipulating and treating it.

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

We will disseminate our knowledge through publications in peer-reviewed journals and presentations at national, international and regional conferences. We will also collaborate with other scientists within and outside of our Home Establishment to progress the work further. We will also share our knowledge with the key stakeholders including patients and patient advocates in patient forums.

Species and numbers of animals expected to be used

- Mice: 600
- 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.

Ischaemia Reperfusion injury (IRI) involves multiple complex biological processes and unknown interactions. To date there is not a suitable non-animal model and therefore the use of animals is the only current method available for the experimental assessment of these interactions. Mice and rats are reliable subjects for surgical work and their use as models of kidney IRI is well known and of widespread use. We have considerable experience with these animals and have developed robust models of IRI using them.

We will use mice and rats at different lifestages - adult, aged and juvenile. Majority of our experiments will be conducted in adult animals as we have conducted previously. We will use juvenile animals in some experiments to gain an understanding of disease and physiological development of the kidneys. We will use aged animals in some experiments to understand how senescence affects kidney injury.

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

The animals will undergo an operation under general anaesthetic. The blood supply of both kidneys will be interrupted for a set period of time and re-instated to create the 'Ischaemia Reperfusion Injury' to the kidneys. The incision will then be closed with sutures and the animal recovered. The animal will then be observed for up to 90 days, before undergoing a terminal operation, at which point the kidneys will be removed and analysed.

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

The animals will undergo an operation to create the Ischaemia Reperfusion Injury to the kidneys, from which one of the adverse effects is postoperative pain. This may be of

moderate severity and will be controlled with effective pain relief. The animals will be observed daily for clinical signs of any distress. One of the other risks is that the kidneys are potentially badly damaged, and the animals may go on to develop kidney failure in the long-term. This is unlikely to be a problem as the animals will be humanely killed at 90 days, well before the potential development of chronic kidney 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)?

The expected severity for all animals will be moderate (100%).

What will happen to animals used in 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?

Ischaemia Reperfusion injury involves multiple complex biological processes and unknown interactions. To date there is not a suitable non-animal model and therefore the use of animals is the only current method available for the experimental assessment of these interactions.

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

In vitro work - namely cell culture and kidney organoids.

Why were they not suitable?

The kidney is a complex organ and kidney ischaemia reperfusion injury involves complex interactions between various cellular process and pathways. An animal model allows for detailed investigations of these processes. Cell culture and kidney organoids are limited in this.

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 we intend to use is based on statistical calculations of the expected differences between different experimental and control groups, for the proposed projects over the next 5 years. The number of animals in each project have been

determined by key findings from previous work that allow for appropriate statistical analyses whilst keeping to a minimum number required.

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

We have used established statistical methods (power calculation based on previous literature and results) and kept the number of animals to a minimum that allows sufficient data as well as reducing harm.

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, a thorough and regular review of results to continue to refine the number of animals needed. Where feasible we will use historical controls. For novel drug studies, we will use dose-response curve to minimise the number of animals and maximise efficiency.

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 performing abdominal surgery to create a moderate kidney injury model. We will give the animals pain relief drugs (in their drinking water) from 24 hours prior to their operation until 2 weeks after their operation. The kidney injury model is designed to provide moderate injury so as to not to have a lasting effect on health.

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

For the research to be clinically applicable, it is important to carry out operations on animals which have fully developed their kidneys. An animal at a more immature stage in life will also be interesting to study for some of the experiments.

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

The rodent is a reliable subject for surgical work and its use as a model of renal IRI is well known and of widespread use. We are experienced with the surgery involved and the facilities are well established within the university to facilitate both the procedures and husbandry of the animals. We will provide the animals with appropriate pain relief, monitor them regularly post-operatively, and will humanely kill the animals well before potential development of complete kidney failure.

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

I have previously visited one renowned establishment to observe their practice in their mouse model of Kidney IRI (unilateral renal pedicle clamping and contralateral nephrectomy) and reviewed literature

from other centres using similar model to us. I have also used my experience as a surgeon and >30 years of experience of my previous mentor. These have allowed me to establish these robust models of kidney IRI. We have recently published a paper describing our models (Robust Rat and Mouse Models of bilateral Ischemia Reperfusion Injury. In Vivo 2024, 38(3): 1049-1057).

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

The Named Persons at the home establishment and the animal care staff are excellent at updating the wider 'in vivo' group about latest advances and developments. These opportunities are carefully considered and where applicable implemented into the project.

70. Farm animal veterinary product testing

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

veterinary, biologicals, pharmaceuticals, livestock, disease

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	juvenile, adult, pregnant, aged
Cattle	juvenile, adult, pregnant, aged
Pigs	juvenile, adult, pregnant, aged
Sheep	juvenile, adult, pregnant, aged
Goats	juvenile, adult, pregnant, aged
Alpaca	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 development and licencing of new veterinary or agricultural products for the prevention and treatment of diseases in livestock and the reduction in the prevalence of zoonotic organisms in the human food chain.

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?

For the prevention and treatment of diseases in livestock it is important to provide good quality, reliable data to the regulatory authorities to facilitate sound regulatory decisions, e.g. on clinical trial approval or marketing authorisation for new medicines or other substances to improve the health and welfare of farm animals. These new veterinary or agricultural products will also help the reduction in the need for the use of antibiotics in livestock production.

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

The project supports the development of safe and effective, new veterinary products to improve the health and welfare of farm animals and provides for safe use of materials to which humans may be exposed in products of food-producing animals.

The outputs of the project will be new knowledge about the livestock diseases and parasites. In the short term we will also obtain information about the efficacy of novel 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 disease and parasite infection in livestock.

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

Farming industry: A key deliverable of this proposal is to determine how to make better and more efficient vaccines, pharmaceuticals and control measures to limit the spread of disease. The beneficiary will be the 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 zoonotic infections will also reduce transmission and the zoonotic threat posed by livestock viruses. Increased productivity in the livestock industry will lower costs for the consumer.

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 diseases in livestock species and humans.

Benefits to policy makers; International development: Development of control measures and better vaccines and pharmaceuticals 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?

Data obtained will be used to obtain market authorisation for biologicals and pharmaceuticals within the EU and globally and therefore will reduce the need to repeat studies.

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

- Domestic fowl (Gallus gallus domesticus): 2600
- Cattle: 2300
- Sheep: 905
- Goats: 205
- Pigs: 3050
- Camelids: 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.

This project uses domestic livestock species, which for the purpose of this project are defined as those species generally regarded as farm livestock, i.e. cattle, pigs, sheep, goats, camelids and poultry. The species of animal selected will be based on the species specificity for the end product required. The life stages that are used are new-born/hatched to breeding adults, including pregnant animals.

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

Animals are dosed/treated by the intended/likely route of target animal exposure, and observed regularly to monitor appearance, behaviour and clinical health. Typical investigative procedures are similar to diagnostic procedures that might be used medically to monitor progress of a human patient (e.g. collection of blood samples for laboratory investigations, or ECG monitoring to assess heart rate/function). Animals will be monitored closely at least 2 times a day.

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. Most animals are expected to experience no adverse effects, or only mild effects such as slight weight loss or occasionally a local reaction at the injection site may be seen, which may result in a small swelling and possible temporary increase in body temperature. A small percentage of animals may show more significant adverse effects indicating moderate severity, e.g. more marked weight loss or reduced activity.

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 are not expected to be of greater than moderate severity. According to the cumulative literature and our extensive experience, 95% of animals are expected to show either no or mild clinical signs. Less than 5% are expected to show moderate clinical signs. Experienced observers, with access to veterinary advice and care at all times, monitor clinical signs of all experimental animals at regular intervals in order to quickly identify any animal requiring veterinary treatment. Any animal failing to respond to treatment will be killed humanely to prevent unnecessary suffering. At the end of the studies animals will be humanely euthanised or for animals involved in on-farm studies will be returned to the farm stock.

The approximate numbers of animals that will be used over the course of this project are:

Species	Number
Cattle	2300
Sheep	905
Pigs	3050
Poultry	2600
Goats	205
Camelids	205

What will happen to animals used in 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?

Non-animal (in vitro, in silico) studies do provide valuable preliminary and supporting data to refine and reduce animal studies, however, 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. Definitive assessments of systemic exposure, efficacy and safety can at present only be achieved in studies using intact animals, and this remains a mandatory legal requirement. Currently, there are no scientifically, ethically or legally acceptable non-animal alternatives available. The EU, FDA, USDA and EPA regulatory authorities require a series of pre-clinical efficacy, safety and residue tests in animals prior to approval of new compounds. These tests are described in regulatory documents.

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

Products that are to be tested will have already undergone evaluation in non-animal alternative tests (replacement) in previous in silico and in vitro studies and that data will be taken into consideration before conduct of in vivo studies.

For example: Cell culture and molecular biology techniques are used to characterise viruses in the laboratory before infecting animals. We will use laboratory techniques to understand the responses of the animals to infection with viruses using blood samples and tissue samples taken post mortem.

New in vitro models are being developed and so 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 virus and disease or parasite infection. Definitive assessments of systemic exposure, efficacy and safety can at present only be achieved in studies using intact animals. Therefore, in vivo studies have to be performed to provide critical data and biological materials that correspond to the outcomes and responses to infections in the animal itself. It is not 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.

The careful refinement of experimental models ensures that only the minimal number of animals required to obtain statistically significant and biologically relevant outcomes will be used. Independent advice on the experimental design is provided by trained statisticians in advance of any experimental work being conducted. In addition, proposed experiments are reviewed by an ethical review committee to ensure that the minimal number of animals is used and that the studies and procedures are justified.

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. Experiments are designed after investigations into published work in the relevant field, discussions with colleagues with relevant experience and, when necessary, the advice of an experienced biostatistician 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. The stated objectives for each trial will be achieved with the minimum number of animals.

Where there are not pre-existing specific models the minimum numbers of animals will be used to ensure the statistical significance of the resulting study data as determined by pilot studies. The NC3R's Experimental Design Assistant and applicable regulatory requirements are used to assist the reduction of animal numbers. Where minimum numbers of animals are specified by regulatory requirements these will only be exceeded to meet the necessary statistical criteria or where a repeat study may be avoided by the use of larger groups.

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 used along side non-animal (in vitro, in silico) studies to provide valuable preliminary and supporting data to optimise reduce the numbers of animals used. Where possible study designs will be combined, e.g. safety and efficacy data will be collected at the same time or cross-over design studies, to reduce the numbers of studies needing to be performed. Controls may be shared between studies where possible.

Tissue and samples, e.g. blood and faeces, will be shared where possible to maximise the use of the products.

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.

- 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

- The severity level after disease or parasite infection is mild in most cases. However, some animals 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.

- Animals housed in the 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?

Products are tested in the target species for any treatment product, the choice of animal is determined by the product use and regulatory requirements.

Rodents do not recapitulate signs of illness observed in livestock; guinea pigs do not exhibit overt signs of illness; and ferrets may have different drug pharmacokinetics to livestock. Only in a natural host such as pigs sheep and poultry, is it possible to dissect the pathogenesis of disease and identify how to control the them.

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

Relevant, reliable and reproducible disease models have been developed and refined to be the least severe necessary for valid results to be obtained. Considerable care and attention has gone into refining the techniques employed to monitor the immune responses during animal studies in order to reduce the degree and duration of any suffering to a minimum. Trained teams of observers monitor animals at regular intervals, accurately evaluating the responses of individual animals and seeking veterinary intervention where necessary.

Sequential testing, with review of findings at each stage and modification of subsequent stages as necessary, maximises opportunities for refinement to achieve the desired scientific endpoints with the least risk of pain, suffering, distress or lasting harm to the animals. Where appropriate, positive reinforcement training (treat rewards) is used to encourage co-operation in (and minimise any stress of) handling/procedures, 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. Environmental enrichments appropriate to the species are used within the animal facilities.

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 livestock 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.

Work will follow published monographs, LASA Guidelines on administration of substances and NC3Rs Guidelines. Other resources that are used are:

- A(SP)A Code of Practice
- DEFRA Code of practice
- RSPCA Welfare Standards
- ARRIVE Guidelines
- The NC3Rs Gateway

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

Regular contact with the NIO and NTCOs from our establishment and from other establishments. I will undertake regular project licence holder training and refresher courses. I will also use other sources of information such as:

- The NC3Rs
- (LASA) Laboratory Animals Science Association

- (LARN) Large Animal Research Network
- (FELASA) Federation of European Laboratory Animal Science Associations
- (ICLAS) International Council for Laboratory Animal Sciences

The Farming events, such as the Pig and Poultry event, are also regularly visited so that new advancements in housing, handling and enrichment can be implemented.

71. Immunopathogenesis and drug resistance in African trypanosomes

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

African trypanosomes, helminths, pathogenesis, coinfections, drug resistance

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?

We aim to understand how trypanosomes cause disease in their mammalian host, in particular how parasite metabolism impacts upon the host response, and how this varies between the genetically divergent trypanosome species that cause livestock disease in the field. We further want to understand how the simultaneous, or sequential, infection of trypanosomes and helminths (a common scenario in the field) in a host might influence the host immune system, severity of infection, or capacity for transmission. Finally, we aim to use our understanding of parasite metabolism to understand drug mode of action, mode of uptake, and mechanisms of resistance - particularly in the species of trypanosome of most relevance to livestock disease, and for both existing and novel 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?

Understanding how pathogens cause disease provides routes to identifying strategies to ameliorate severity. How parasites generate the energy to survive and multiply, and what they consume and produce in these processes, has many impacts upon and consequences for the host - including on how the immune system functions. Parasite metabolism is also central to how drugs act, and to the mechanisms that parasites develop

to become resistant to treatments - this is particularly true for trypanosomes, where most identified drug resistance mechanisms are involved in metabolism. For trypanosomes, most of what we understand in these areas derives from the well studied causative agent of human disease, Trypanosoma brucei. However, livestock disease is predominantly caused by two other, very genetically divergent, trypanosome species, T. congolense and T. vivax. It is increasingly clear that this genetic divergence results in significant phenotypic divergence, including with respect to important phenotypes such as fundamental metabolism, disease severity, mechanisms of immune evasion/immunosuppression, and drug mode of action/mechanisms of resistance. It is vital to understand the diversity of disease manifestations that are driven, and how they are caused, by this genetic divergence, as well as how the genetic divergence translates to variation in drug susceptibility and ability to develop drug resistance. Therefore, most work to be carried out under this license aims at dissecting how the genetic differences between these parasite species translate to differences in pathogenesis, drug or vaccine susceptibility, and propensity to develop resistance to both existing and new drugs. This ultimately has the potential to inform the development of novel interventions (in particular drugs and vaccines), as well as how best to use existing and novel interventions to combat the diversity of trypanosomes causing diversity in the field. Finally, a very common manifestation in the field (in human or livestock disease) is coinfection of trypanosomes with intestinal helminth parasites. It is well established that helminth infections can impact upon the immune responses to either vaccination or infection with other classes of pathogen. However, remarkably this is almost completely unstudied in trypanosomes. Understanding the interactions of the host with trypanosomes and helminths in coinfections could have important implications for disease severity, transmission and wider disease epidemiology, and also potentially the efficacy of treatment or vaccination targeting either pathogen.

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

Trypanosomes are complex organisms, and our understanding of which of the many host-pathogen (and pathogen-pathogen) interactions are critical to causing disease is still very limited. We have very few drugs and no vaccines to combat the disease in livestock or humans, and a greater understanding of parasite infection biology provides a route to identifying strategies for disease intervention (e.g. drug or vaccine targets). Additionally, given we have so few drugs to tackle this disease, and the use of the available drugs is very widespread, resistance is a significant problem. However, we understand very little about how the trypanosome species that cause livestock disease become resistant - identifying how trypanosomes become resistant enables the design of drug use strategies that can mitigate resistance arising, and the identification of markers enables the measurement of resistance spread in the field. The work will generate research publications for the scientific community but will also be disseminated to the public through appropriate channels (e.g., newspapers, radio and television).

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

The primary and short term beneficiaries will be the scientific community that is working on trypanosomes and the disease that they cause. However, some aspects of the work (e.g. drug or vaccine candidates, understanding implications of co-infections on disease outcome, knowledge on how parasites become resistant to particular drugs, and markers for such resistance) may be of benefit to others in the short to medium term - for example, policy makers such as the Food and Agriculture Organisation are designing international strategies to combat the spread of drug resistance for which such outputs may be directly useful; similarly pharmaceutical companies are developing the first new drugs to combat

animal trypanosomiasis in over 60 years, and understanding how and how quickly trypanosomes become resistant to the drugs in development can inform how new drugs are best used in combination with the few existing therapies in order to maximise the useable lifetime of all available drugs in the armoury.

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

Our laboratory collaborates widely with researchers in the UK, Europe, USA, South America, and particularly extensively across Africa. These activities generate knowledge sharing and joint publications, personnel exchange, etc. We have been particularly active in this area, establishing a network of researchers active in livestock trypanosome research in order to share findings, protocols and reagents - this has been stimulated by two symposia on African Livestock Trypanosomes involving researchers, funders, NGOs, national and international control programmes, pharmaceutical companies, and government representatives. In addition to publication in high impact journals, we have (and will continue) to publish less impactful or negative outcomes in specialist research journals as well as general open access avenues. We have also put considerable effort into communicating our research through the publication of a comic aimed at secondary school children ("more milk Zuri"), which has been widely disseminated at public engagement activities locally and across Africa, and this has been developed further into an interactive card game that is used in schools' outreach, participation and engagement days and activities.

Species and numbers of animals expected to be used

- Mice: 2000
- 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 will predominantly use adult mice. Mouse infections allow comparison with previous analyses of routine infections in our laboratory (and other laboratories); infections in these animals are very reproducible and predictable over the first wave of parasitaemia, which will be the focus of most of our studies. However, some studies will use longer term infections to explore the consequences of the chronic stages of infection, including the impact upon trypanosomes of co-infections with helminths (to mimic a very common scenario in the field). Some experiments will use particular mouse strains where this is necessary for comparison with results from other labs that use that model. Our lab has many years of experience of monitoring trypanosome infections in mice and so are able to identify the likely progression of parasitaemia and associated clinical signs. Work involving helminths uses a species that is well established as an infection model, and induces mild pathology, and that in wild type mice are self limiting resulting in pathogen clearance. By accurate projection of the course of infection, monitoring can be increased as necessary during critical phases of infection. The predictability of infections will also aid the selection of appropriate time points to capture parasites at different stages of differentiation. It also minimises the possibility of unanticipated infection pathology ensuring that experiments derive maximum outputs from the minimal number of animals used.

Rats are used when larger numbers of trypanosomes are required- approximately 10 times as many parasites can be harvested from adult rats as from adult mice.

Although in vitro culture of parasites can be used for many experiments, some strains of the livestock- infective species (T. congolense and T. vivax) cannot be cultured presently, and need to be grown in mice. Additionally, analyses are required where the context of infections in vivo are being explored, for example in the presence of the host immune system or where interactions with co-infecting helminths are being studied. Furthermore, the number of trypanosomes that can be isolated for molecular studies from infections is much higher than from culture, such that in vitro methods are not possible for some analyses and become unfeasible where large amounts of parasite material are required for study.

Hence, infection-derived material can provide sufficient material to make feasible some molecular purifications that are impossible using culture methods and this is necessary to achieve the scientific objectives of the work.

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

Typically, animals will be inoculated via the intradermal, intraperitoneal or intravenous routes with trypanosome parasites, optionally after their immunosuppression with cyclophosphamide. The infections will then be monitored by sampling the tail vein by scab removal after around 3 days until the first peak of parasitaemia occurs in wild type parasites (typically after 6-9 days). Where molecules potentially important in pathogenesis or drug/vaccine susceptibility are identified, mutant parasite lines will be created and their virulence and progression through infection will be monitored after ectopic expression, gene deletion or gene silencing, this being carried out with appropriate group sizes and in comparison to wild type parasites.

Many infections will be terminated after 6-9 days at first peak parasitaemia to enable isolation and analysis of parasite and host factors. For some experiments longer term infections will be analysed using parasites that establish chronic infections. These infections will be monitored for the kinetics and developmental status of trypanosomes, as well as host immune response factors.

For helminth infections, animals will be inocluated via oral gavage with well established experimental model species such as Trichuris muris or Heligmosomoides polygyrus, each of which can establish chronic infection without inducing overt clinical signs in the mice (and in the case of Trichuris muris infections are normally cleared after ~3 weeks by wild type mice). Mice will be monitored for weight loss (and potentially egg production in faeces), normally over a period of 4 weeks.

Coinfections will involve either simultaneous or sequential infection of mice with trypanosomes (ID, IP or IV) and helminths (oral gavage), with parasitaemia and health parameters monitored as described above.

The distribution of parasites within the mouse host may also be monitored using red-shifted luminescence imaging under anaesthesia. This involves the inoculation of parasites IP, and then the monitoring of infections at time points after infection under anaesthesia by the inoculation of a luminescent substrate to allow the compartment or distribution of the parasites to be visualised using recording equipment.

Where pharmacological agents are identified that may alter the development or viability of the parasite, these will be investigated for their effects on parasite viability, development or

growth in vivo. This will be preceded by range-finding experiments where appropriate pharmacological doses are determined. In each case, parasite numbers will be assessed by isolation of a blood drop from the tail vein and then analysis of parasite numbers or morphology microscopically or using molecular markers (e.g. immunofluorescence microscopy, qRT-PCR).

The transmission capacity of parasites may also be monitored by assessing the ability of tsetse flies to become infected after feeding on anaesthetised infected mice.

In all cases, at the end of experiments, animals will be terminally anaesthetised and, optionally, parasites harvested by cardiac puncture and blood collection in the case of trypanosomes, or dissection from the relevant intestinal niche in the case of helminths. Organs might also be isolated post mortem to determine the distribution of parasites according to the luminescence of tissues infected with parasites expressing red-shifted luciferase.

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

Trypanosome infections can be fatal in rodents, death being preceded by progression through a predictable series of symptoms on a relatively predictable timescale. Our experience of working with these parasites in mice (over 20 years) means that we have become expert at monitoring and predicting the progression of infections, so that experimental outcomes can achieved without the infection leading to severe illness or death, which is a rare occurrence (less than 0.5% of infections). The helminth species to be used cause little or no pathology in the mice, but a well established monitoring system (based on weight and behaviour) will be used in case, for example, coinfections with trypanosomes or genetically modified mouse strain, result in any exacerbation of symptoms. Mice and rats are monitored for their disease progression based on a numerical scoring system and undue suffering prevented by humane killing should the infection progress to a level where death is anticipated within a few hours or might occur if animals were left unmonitored overnight. As a consequence, the overall severity for our experiments is classed as moderate. At the end of experiments animals are euthanized and parasites are harvested.

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- expected severity based on values from 2019

Mild- 32%

Moderate- 68%

Rats- expected severity (values are estimates based on historical values)

Mild- 50%

Moderate- 50%

What will happen to animals used in 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 routinely grow trypanosomes in cell culture. However, not all strains or species of trypanosome are able to be cultured, and therefore in many cases amplification of healthy parasites is best achieved by growing parasites grown in mice. Furthermore, the evaluation of the effects of disrupting particular parasite molecules and processes that impact upon the host immune response can only be accurately determined by studying parasites growing in the natural context of a bloodstream infection in a mammalian host, where the host physiology and immune response may contribute. For the isolation of large numbers of parasites, the ability to harvest tens of millions of parasites from a single infection makes some experimental approaches feasible that would not be possible using cultured parasites that are in an un-natural growth medium and isolated from the host (and thereby in the absence of an immune response and where molecular signals are not at natural levels due to their non-physiological accumulation or turnover in culture). Coinfections between trypanosomes and helminths also require to be studied in the context of a functional immune system. Whilst interactions between parasites in culture can be informative, gastrontestinal helminths cannot currently be propagated through their life cycle in vitro, and therefore ultimate validation of any effects observed needs to carried out using parasites growing in a mammalian host where the combination of immunity, signal production and turnover can contribute to the infection dynamic. Where parasites occupy particular host body compartments (e.g. trypanosomes in particular tissues, or helminths in particular areas of the intestine), this must also be assayed in vivo.

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

We have developed non-animal based protocols for growing parasites in vitro, through assessing what metabolites parasites cosume and produce - enabling improved in vitro culture for the mammalian forms of particular strains of the livestock trypanosomes T. congolense and T. vivax. These are effective at generating reasonably large populations of cells of these particular strains, which allows the generation of parasite materials (e.g. DNA/RNA), analysis of drug/other molecule sensitivity, and the genetic manipulation of these parasite lines, including the evaluation of such genetic perturbations on some basic parameters (e.g. rate of growth, survival in presence of drugs/other molecules). However, these assays cannot fully recapitulate growth in vivo, and so experiments must also be validated in vivo or extended to assay the contribution of the host physiological environment. There has been a lot of work to pioneer and develop non-animal based models for working with helminths - in particular intestinal organoid systems - but these are currently not at a level that supports completion of the life cycle of the target helminth species, and the culture media used for organoids does not support growth of trypanosomes, and therefore coinfection studies with trypanosomes and helminths cannot currently be undertaken in vitro.

Why were they not suitable?

In vitro culture cannot accurately reflect the complexity of in vivo signals, spectrum of host cell involvement, or the variety of parasite interactions with host molecules or the immune

system, or the indeed kinetics of molecule accumulation/turnover (e.g. drug, virulence factor) in vivo. In addition, the contribution of physiological compartmentation (e.g. in the adipose tissue or skin) cannot be studied in vitro. For these reasons assays finally must be carried out in vivo to assess the environment in which parasites establish and differentiate accurately.

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 based on previous laboratory usage and reflect the number of animals needed to generate molecular material or maintain parasite blood stocks (e.g. of parasite species/strains that cannot be cultured in vitro), or the number of animals required for statistical validity when assessing phenotypes after gene (host or parasite) knockout, knockdown or overexpression studies. Likewise, experimental design considerations for the analysis of coinfection experiments dictate the numbers of animals used in experimental groups. In all cases, pilot assays are also used to establish the dose of trypanosomes or helminths for infections, their likely progression of growth and differentiation and dosages for particular pharmacological regimens. These small pilot studies help to optimise the experimental studies where larger groups of animals are used for statistical analysis. Maintenance of trypanosome blood stocks (which can be cryopreserved) can also use small animal numbers.

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

Trypanosome growth in mice progresses on a relatively predictable course, such that it is straightforward to generate reproducible and statistically valid datasets that satisfy external scientific scrutiny (i.e. where the probability of the observed outcome being incorrect is less than 5%). We are experienced in analysing and predicting parasite virulence in mice and so can minimise distress whilst deriving the necessary scientific information from infections. Genetic manipulations of trypanosomes often involve use of gene silencing or gene overexpression techniques, which are controllable using chemicals supplied in the rodent drinking water (e.g. doxycycline). This provides well controlled analyses because phenotypic comparisons between treated and untreated populations provide a robust experimental outcome using the same parasite material. We can access the assistance of a statistician to help plan appropriate group sizes for studies where there is variance in the effect size, providing advice on the planning and interpretation of experiments to achieve statistically meaningful outputs.

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

Pilot assays are used to establish the dose of trypanosomes or helminths for infections, their likely progression of growth and dosages for particular pharmacological or experimental regimens. These pilot studies help to optimise the experimental studies where groups of animals are used for statistical analysis.

Relevant in vitro assays also optimise our studies by identifying those cell lines that exhibit the appropriate gene perturbation or best regulation using inducible systems such as tetracycline/doxycycline controlled overexpression or gene silencing.

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 used to monitor trypanosome infections in laboratories worldwide. This allows comparisons of the infection profile and kinetics of proliferation and differentiation between studies and between laboratories. The symptoms linked to trypanosome infection in mice are also predictable allowing us to track the progression of infections with a scoring system allowing defined humane end-points. Notably, in most cases we are also using trypanosome strains (T. brucei AnTat 1.1, T. congolense IL3000, and T. vivax Y486) that are used my multiple laboratories across UK and Europe in animal studies, helping assessment of reproducibility and aiding comparisons across studies - the same is true for Trichuris muris and Heligmosomoides polygyrus, where we use isolates that are used by mutiple laboratories across the UK. A staged approach will be adopted when novel drugs are used. Mice do not exhibit overt signs of distress or discomfort from trypanosome infection except in the critical phases when infections are closely monitored.

Rats are required occasionally where it is important to isolate very large amounts of parasite material - beyond that achievable either in vitro or in mouse infections.

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

Trypanosomes can only infect mammalian hosts as their disease relevant bloodstream form, and helminths are similarly host-restricted. Infections must be sustained over a period of days to allow for parasite development, or longer to explore aspects of the parasite's interactions with the host or other parasite strains or species. Adult animals are required to explore the parasites' biology in a manner relevant for livestock or human infections in Africa.

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

We carry out ongoing review of procedures, in consultation with the relevant services at the Institute, to assess where animal welfare can be ensured and enhanced. Examples of procedure adaptations include improved environmental enrichment, avoiding single housing where possible and exploring the potential for the introduction of refined local anaesthesia for the harvesting of small parasite samples from the tail vein etc.

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

Experimental procedures have been developed in house but also adapted or adopted as new approaches are described or detailed in the literature. For example, we have published methodology for improved ability to culture trypanosomes in vitro (in particular the livestock species T. congolense and T. vivax). Other methodologies follow those of colleagues in other laboratories.

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

Ongoing training and education will be provided through the Institute, with updated information provided via their regular community meetings and information updates. Training guidance will be disseminated through local animal unit personnel and at our own laboratory meetings, held weekly.

72. Defining how novel macrophage receptors influence development of ovarian cancer

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cancer, macrophage, metabolism, cholesterol

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 define how novel receptors expressed by macrophages control the behaviour of these cells 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. Understanding the early stages of this cancer will help us better diagnose the disease, which would mean that available treatments could be given earlier and have a greater chance of curing the cancer. Currently available treatments for ovarian cancer have lots of side effects, therefore we need treatments that are more specific, and we can only develop these new treatments if we first understand how the cancer develops and why the immune system is unable to kill it. A type of immune cell called a macrophage has been shown to promote the progression of this cancer, but exactly how macrophages do this isn't well understood. We have identified genes that are highly expressed by macrophages in ovarian cancer patients, and where the higher the expression of the gene correlates with the likelihood of death. We want to understand why this is, and whether these genes expressed by the macrophages could be targeted for therapy.

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, in addition to answering our scientific questions on the role for macrophage metabolism, our results will still be useful to others and worthwhile reporting as new findings or as a published protocols and refinements.

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 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. Mice are particularly useful for this since most genes have now been targeted or altered in mice, unlike other model systems (e.g. fish) where genetic alteration studies have not been as widely utilised. Our analysis of genetic data from ovarian cancer patients indicates that macrophages (an immune cell) in the tumour highly express genes that encode receptors and enzymes 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 experiments will aim to examine how the function and phenotype of macrophages changes when these genes are deleted during ovarian cancer, and whether these changes have any effect on the progression or clinical presentation of the cancer in the early stages.

2) We can study the very early stages of ovarian cancer in mice. One of our hypotheses is that macrophages in the omentum and peritoneum (the places that cancer cells spread to and cause disease in humans) 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 enter these locations.

3) Mice have highly similar immune systems to humans, hence they are an excellent model system to study immune responses in disease contexts in which the immune system plays a key role in pathogenesis. Macrophages form specialised subsets and generate via developmental pathways that have been found to closely mimic what occurs in humans. Mice are therefore a relevant species to study macrophage function and phenotype in cancer, where macrophages have already been demonstrated to contribute towards metastasis and poor outcome in cancer patients.

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 (either into the ovary or the peritoneal cavity) 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 examination of overall body condition. 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 end the experiment 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). Moreover, this approach will reduce the number of animals we need for our studies overall by producing tighter data with less variability as we can analyse tumour development in individual mice over time. At various time-points after injection of cancer cells, we will humanely kill the mice and isolate macrophages from various body sites (e.g. omentum, ovary, peritoneum) and analyse them for markers of pro-cancer pathways and/or pro-inflammatory functions that can help kill tumours, comparing between wild-type mice and those with genes deleted that we hypothesise are involved with macrophage function in cancer. In some experiments, we may inject drugs that delete macrophages so that we can assess how macrophages contribute towards cancer spread. Drugs that delete macrophages are not expected to cause any adverse effects or symptoms in mice.

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 (which includes consideration for overall body condition), for up to 5 weeks. Mice may experience increased 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) at which point animals will be humanely killed 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)?

~45% sub-threshold (used only for breeding or tissue collection without procedure)

~5% mild severity

~50% moderate severity

What will happen to animals used in 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 function 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 or peritoneal cavity are different to the signals they receive in the ovary, and this changes how the macrophages function. Some of the latest data has shown that macrophages in the peritoneal cavity drastically change their phenotype once exposed to ovarian cancer cells. This in turn seems to change how the cancer cells then invade other body sites, such as the omentum. The receptors that control these changes in the macrophages aren't known. To be able to test which receptors and pathways are most important for the detrimental functions and changes of macrophages in the context of ovarian cancer, we need to be able to compare macrophages from different sites (e.g. peritoneal cavity, omentum, ovary) in the presence or absence of the macrophage receptors of interest, and at the same time look at how the ovarian cancer cells move around the body (e.g. invading the omentum, the main site for metastasis in ovarian cancer). 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 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) Publicly-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, and how this correlates with mortality and disease outcome in patients. We have used this approach to identify the receptors and enzymes expressed by macrophages that might be important for cancer development, which have helped us narrow down the types of transgenic mice we will use for this project.

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.

4) Organoids. We are currently exploring the use of ovarian cancer organoids for use in this project. These are 3D cultures of human ovarian cancer cells. We are beginning to expand these organoid lines from different patients, and we will then determine whether macrophages can be incorporated into these cultures to help us answer some of our research questions (such as how the environment within the ovarian tumour may affect macrophage phenotype or function).

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 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 tumour or peritoneal cavity. 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?

Where available, we will use in-house data (generated from studies completed under our other licences) as part of our calculations required to estimate the number of animals used. Where this is not available, we will instead use published data from other groups or data from our collaborators.

Where there is no pre-existing data available, we will perform a pilot study to establish baseline data upon which to base a calculation.

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 inflammatory phenotype and metabolic indicators from a number of different tissue sites (e.g. ovary, omentum, peritoneal cavity) which will help us determine how different organs change macrophage function (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.

Lastly, we will use cancer cell lines that express luciferase in some of our models, which allows for non-invasive imaging techniques to be employed to examine tumour spread and development. This means that the same animal can be analysed at multiple time points over the course of experiment, significantly reducing the total numbers of mice needed to answer some of our scientific questions. 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 and before development of adverse effects associated with more advanced disease.

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 two different routes to model ovarian cancer in this project, each with their own distinct advantages and scientific aims.

Model 1: Intrabursal injection to model primary tumour development - In this model, 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. Cancer cells may then spontaneously migrate from the primary tumour into the peritoneal cavity and omentum. This dynamics of this will depend on the cancer cell line used, the mouse background, and dose/number of cells injected. This model is not well used in the field currently, with only a few studies publishing data from it so far. However, this model appears to better mimic clinical progression of cancer. This is important because many pre-clinical studies on immune responses during cancer do not translate well to humans because they do not mimic natural progression of cancer and/or do not study primary tumours at relevant sites. This model circumvents these issues and is more likely to generate data that will be of higher relevance to human disease.

Model 2: Intraperitoneal injection to model early macrophage changes - Intraperitoneal injections of cancer cells results in rapid changes to the macrophages within the peritoneal cavity and omentum, and can enable analysis of the earliest changes occurring within the macrophage population prior to the development of large tumours or other side effects (e.g. ascites). We will use this model to identify and characterise phenotypes in our transgenic mice at the earliest time points after injecting cancer cells, helping us to narrow down what macrophage changes to focus on in the more clinically-relevant intrabursal model, as well as identify mouse lines that should not be taken forward into the longer intrabursal model (e.g. due to a lack of phenotype in the faster intraperitoneal model). Lastly, this model gives more consistent cancer cell growth in the omentum, the main site of metastasis in ovarian cancer and where macrophages are thought to promote cancer cell growth. In the intrabursal model, cancer cell spread to the omentum is likely to be more variable between mice. Therefore, the intraperitoneal model enables specific examination of the omentum and the macrophages that reside there, helping to answer some of our key scientific questions.

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 and the study is too long to use terminally anaesthetised 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 further develop a refined methods of monitoring tumour growth and spread. For example, we plan to use a luciferase-expressing cancer cell line and imaging, which will reduce the number of mice needed to gain results, but it also presents an opportunity to refine the model. Whilst the repeat imaging will mean animals undergo multiple anaesthesias, any negative impact of this is minimal compared to the benefit of early detection of rapid tumour growth prior to the onset of clinical or outwards symptoms (e.g. swollen abdomen indicating early stages of development of abdominal fluid). We will also correlate our these 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, resulting in a high quality clinical scoring sheet that can be used for our other studies, and that by other groups, in future work.

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

There are numerous published studies that we will use to inform our choice of cancer cell line used in the models and the timelines used for our studies. We will also refer to ARRIVE 2.0, LASA, NC3Rs and PREPARE guidelines in the design of our experiments.

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.

73. Targeting cancer metabolism and boosting the immune system to reduce cancer burden

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 metabolism, Immune response, Resistance, Metastasis

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 overall aim of this project is to identify therapeutic interventions that will target cancer cells directly or boost the immune system to prevent cancer establishment 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?

Cancer is going to become the world’s leading cause of death within a few decades and we are currently ill-equipped to deal with this global crisis.

This disease already kills more than 10 million people every year and 90% of the deaths are caused by drug resistance: strategies to help increase survival should rely on prevention and early detection, but also new treatments, especially drugs targeting the therapy-resistant and metastatic disease.

It is key to develop new therapeutic interventions to reduce cancer burden, focusing in particular on treatments that target cancer metabolism or boost the immune system.

We seek to obtain key pre-clinical findings that will then be translated into clinical studies with the ultimate aim of establishing new therapies and we will focus in particular on gynaecological cancers, lung cancer and melanoma, which all together are responsible for almost one fourth of all cancer deaths.

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

We will identify the potential of several compounds to be clinically effective in reducing cancer burden. We will also establish whether these drugs work better with already existing treatments such as chemotherapy and immunotherapy, without damaging the surrounding normal tissue, to fight cancer. We would anticipate further clinical trials with these novel combinations.

We will also aim to understand the mechanisms involved in the tumour response, to potentially open new therapeutic avenues.

The results of the proposed in vivo pre-clinical studies will be made available to the scientific community through presentations at national and international conferences, and publications in peerreviewed journals. Where applicable, the results will also be made available through publicly accessible databases, and the outcomes of this project will be highlighted through the relevant funding bodies.

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

In the short to medium term, we expect other scientists with an interest in cancer therapies to benefit from our findings that we will publish in relevant journals or present at conferences.

In the medium to long term, possibly after the life time of this project, we expect clinicians and cancer patients to benefit from the development of new therapies that we hope to take to clinical trials.

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

The results of this work will initially be presented to the scientific community through

National/International meetings. The meetings will be carefully selected and will include both smaller specialised conferences, as well as larger international meetings in order to reach both basic research and clinical research audiences. The data will subsequently be published in high-impact journals with relevant open access provision.

Species and numbers of animals expected to be used

- Mice: 4,300

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.

We are using adult mice because they represent a faithful model for the malignancies we are mainly focusing on (gynaecological cancers, lung cancer and melanoma); these diseases almost exclusively affect adults and their patterns of tumour growth in humans can be mirrored in adult mice. Also, the knowledge on cancer related protocols and responses to therapies in mice is broadly available.

We may also use genetically altered mice, specifically strains of mice lacking particular components of the immune system that allow us to test the implantation of human cancer cells. These genetic alterations do not harm the animals and their use is important because they allow us to study human disease in mice and later translate these results into humans.

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

Some animals will have tumours induced by the injection of cancer cells using either the subcutaneous route, which is typically into the flank of the animal, intraperitoneally or intravenously.

Tumour growth will be measured by non-invasive methods such as callipers or 3D scanning which involves injection of an agent to facilitate detection of the cancer cells and short term restraint for the animal; in other cases the animals will undergo repeated imaging by methods such as MRI, under general anaesthesia. The imaging methods are like those a human may undergo to assess tumour detection or progression.

Some animals will then be treated with compounds such as existing or novel therapeutic agents that may treat cancers or boost the immune response. They may receive up to two compounds to enable us to see how the drugs react in combination.

Some animals will be fed a diet where the fat content has been modified. This may be a higher or lower fat content. This is to allow us to assess the influence of diet on the compounds' concentration in the blood.

Blood samples will be taken on occasions to allow us to detect the presence and measure the concentration of our drugs.

Some animals will receive substances that will mark cells prior to humane killing.

At the end of the experiments all animals will be humanely killed, with tissues and organs harvested post-mortem for further analysis in the laboratory.

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

Genetically altered mice that lack particular components of their immune system will be housed in bio secure caging systems to avoid infections.

Subcutaneous tumours may invade the overlying skin or muscle which may impede normal movement. Some tumours may ulcerate. In most cases this will be seen as a dry scab but in some cases a wet ulcer may develop which will either be treated under veterinary advice or humanely killed if there is no sign of healing within 48 hours.

Intravenous tumours may result in metastasis and cause tumours to develop in the lungs resulting in laboured breathing. Animals will be immediately humanely killed if laboured breathing is detected.

Weight loss is also possible in the later stages of tumour growth of up to 15%.

As with humans, some treatment for cancer may result in dehydration, weight loss of up to 15%, and diarrhoea.

Diets with a higher fat content may cause the animals fur to become greasy and result in some soreness of the skin.

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)?

70% mild, 30% moderate

What will happen to animals used in 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 order to fully understand the interplay between cancer cells and the components of a whole living mammal, in particular the role they play in the response to anti-cancer drugs. In some experiments, we will need to have a fully functional immune system, while in others we will focus on the tumour microenvironment (adipose tissue, fibroblasts, etc). Mice are the least sentient species that mimic the human physiology.

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

As a non-animal alternative, we extensively work with bi-dimensional and three-dimensional cultures of cancer cells. These models recapitulate some features of solid tumours, like the concentration of oxygen and nutrients and, therefore, are useful in the context of metabolism research. In our lab, the use of these models enables us to select a narrower range of suitable candidate compounds for in vivo evaluation and considerably reduce the number of experimental animals.

Why were they not suitable?

Whilst cell cultures are a useful tool to preliminary screen anti-cancer compounds, they are not able to adequately replicate the full metabolic processes that underpin cancer in a living organism. Moreover, we are interested in the role of the tumour microenvironment during cancer treatment, which can almost exclusively be studied in the whole 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?

Our estimations are based on similar studies and our previous knowledge on the variability of the outcomes of interest, for the specific animal models used 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 use a variety of strategies to ensure that the minimum number of mice necessary to achieve the scientific objectives is used in each experiment. We have implemented the NC3Rs experimental design guidelines to design our experiments, for example by ensuring randomization and blinding to avoid bias and increase reproducibility. We use data from pilot experiments in power analysis and statistics-based guidance for future experiments. For example, we determined the effect size from our collaborators' previous experiments using the mean time to reach a specified tumour size as endpoint. This effect size was then used to calculate the sample size required in our proposed experiment to allow robust comparison between groups. This gave us the overall number of animals included in this project. With collaborators, we have also performed pilot studies testing some of the compounds in combination with common chemotherapeutic agents, which will reduce the number of pilot studies and animals to be used in this project.

In order to minimise variables and ensure reproducibility we apply a sturdy methodology in our lab, including the implementation of Standard Operational Procedures for numerous procedures, for example tumour cell culture, preparation and injection, preparation of compounds and vehicles, and their administration to experimental animals, as well as tumour measurement.

Robust methodology also includes the use of appropriate controls in each experiment, so that conclusions are sound. Each experimental group will differ in only one variable with respect to the correspondent control group, so any differences would be attributable to such variable.

Housing of experimental animals also plays an important role in experimental variability. The risk of opportunistic pathogens to immunocompromised animals is significantly reduced by the use of bio secure caging and enhanced husbandry routines (e.g. using irradiated feed and autoclaved bedding).

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 to assess new variables such as novel compounds in combination with known compounds such as common chemotherapeutic agents.

This will also enable us to further investigate the tumour response and determine the dosing regimens required for chemo or immunotherapy in a reduced number of animals. Data from these pilot studies will include information on tumour growth rate and kinetics, compound levels in blood, immune profiling and other physiological parameters; this will provide the details we need to determine the smallest number of animals required to be of statistical significance.

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 will be used, as they are the best characterised species to assess tumour responses. More specifically, we will be using two main models: 1) immunodeficient mice for studies with xenograft tumours (i.e. when the cells that are injected in the animals to form the tumours come from human samples); relying on these types of mice will improve the animals' experience, as they are less likely to suffer from rejection sickness. We will use immunodeficient mice that have been genetically modified based on their ability to generate the amount of immune response required for our scientific outputs. 2) immunocompetent mice when we wish to create allograft tumours (i.e. the cells that are injected come from another mouse with a different genetical makeup); these types of mice are the most refined because they are less prone to infections, while from a scientific point of view they allow us to study the immune response of the animal and test our anti-cancer drugs in combination with immunotherapies currently used in the clinic.

In both immunodeficient and immunocompetent models, we will be inducing tumours through a subcutaneous injection of cancer cells. This method of inducing tumours is considered to be more refined, as the animal welfare is not greatly compromised, since it spares normal organs. In some instances (for example when looking at lung metastases or ovarian cancer metastatic spread), we will have to use intravenous or intraperitoneal injections respectively, because these methods better mimic the metastatic patterns observed in patients.

Tumours will be measured through callipers or scanners. This will involve handling the animals with the least amount of stress for the animals as possible.

We want to test novel compounds to be able to assess treatment. To minimise the adverse effects of any novel compounds administered, these will be first tested using small quantities to identify the nontoxic doses and best routes of administration. Blood samples will be taken on occasions to allow us to detect the presence of our drugs and ensure we are using the most clinically relevant and non-toxic doses.

Some animals will be fed a diet where the fat content has been modified: it is known that in some instances our compounds' uptake can be enhanced by increasing the fat content in the diet, which in turn would reduce the number of doses of compound to be administered and therefore represents a refined administration method. This modified diet will be maintained for the duration of the treatment, returning to normal diet thereafter.

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

Mice are classified as the lowest order species in which we can study tumour growth and adaptive immunity in a way that is relevant to humans. In less sentient species, such as fish or worms, both the immune system and the tumour microenvironment are too different from that of humans; therefore, research conducted on these animals would hardly lead to any clinically translatable results.

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

We will follow published tumour guidelines and have monitoring and controlling measures in place to minimise the harms to the animals. For compounds being tested, we will use the minimum dose that will allow us to reach the scientific output. To minimise discomfort to animals, whenever possible the least invasive route of administration will be chosen. If

intravenous injection is needed for compound administration, both lateral veins will be used to minimise the risk of vein damage. We will also supply food and water at cage at floor level where the tumour has invaded overlying skin and muscle and therefore restricting movement to minimise any stress for the animal. They will also be more closely monitored.

Mice will also be weighed at least twice per week and if weight loss of 10% is seen, moist palatable food will be provided to arrest the decline in weight. Mice exhibiting weight loss will be checked at least once per day.

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

We will follow:

LASA guidelines for best practice in volumes and routes of substances.

Published papers that show guidelines for the welfare and use of animals in cancer studies.

NC3R's for both procedural and husbandry best practice.

ARRIVE and PREPARE guidelines to ensure experiments are reproducible and printable.

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

We will sign up to the NC3R's newsletters and regularly check the website. We attend events nationally as well as local events at our establishment where the 3R's are regularly discussed.

We will also rely on our named information officer.

74. Biology & genetics of Strongyloides parasites

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Parasites, Strongyloides, Neglected Tropical Disease, Infection, Helminth

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?

Understand the biology and genetics of the gastrointestinal parasite *Strongyloides.*

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?

Soil-transmitted helminths, such as the parasitic nematode Strongyloides, are categorised by the World Health Organisation as a Neglected Tropical Disease and are estimated to infect approximately 1.5 billion people globally. It is important to study these parasites and understand the underlying biology that makes them a parasite to improve control, treatment and diagnostics.

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

Expected outputs of this project include (i) new information about the molecular and genetic basis of Strongyloides parasitism and host-parasite interactions, which will be disseminated through peer- reviewed publications and conference presentations, (ii) large data sets including gene expression information, genome assemblies and annotations and small RNA sequences which will be made publicly available through appropriate data repositories.

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

This work will advance fundamental scientific knowledge about how parasites infect their hosts. Other researchers including those in the fields of parasitology, genetics and nematode biology will benefit from the information we produce in publications.

We will generate large datasets that will be made publicly available and will directly benefit other researchers who can use these data for further analyses. These data could be developed and used to improve diagnostics, treatments and control strategies of Strongyloides infections e.g. by targeting or further investigating key molecules we identify.

Therefore, humans and other animals infected with Strongyloides parasites are the ultimate long term beneficiaries.

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

Outputs from this work will be maximised through dissemination of new knowledge through open access peer reviewed publications. Publications will be made available through pre-printer servers such as Bioxiv where possible to enable early access to the information.

Results will further be disseminated through conference presentations at scientific meetings at national and international levels. Established and new collaborations will be built upon to further explore and share the resources, knowledge and data generated from the project.

Species and numbers of animals expected to be used.

- Rats: 4240 wild type rats, 560 GA 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.

To achieve the aims of the project i.e. to understand the genetics and biology of the parasitic nematode Strongyloides, Strongyloides nematodes will be studied in the laboratory. To do this the Strongyloides nematodes need to be maintained in vivo in rats.

This is the only way that Strongyloides material can be obtained for these studies i.e. by maintaining the life cycle of these parasites in a host animal. There are currently no alternative methods for maintaining Strongyloides nematodes in the laboratory that do not involve the use of animals.

Rats aged 4 weeks and older will be used to maintain Strongyloides infections. We use rats because they are a natural host of the parasitic nematode. Using the natural host is important because it means that we will study the natural infection process that has evolved. During infection no noticeable harm is caused to these rats and eventually the rats become immune to the nematode infection.
We will also use GA rats such as nude rats and rats with other immune or infection

response- associated deficiencies. GA rats will include rats deficient in aspects of the Th2 immune response, the immune response most commonly associated with helminth infections. The use of GA rats will enable us to better understand how the parasite interacts with its host.

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

We will use doses and routes of infection that best mimic the natural route of infection, and that cause minimal harm to the animals to achieve the scientific objectives. The parasite-rat system is well established and collective experience from this field of research has provided established protocols which minimise clinical symptoms. Where genetically altered nematodes are used these are expected to be less effective parasites than wild types strains. However, as a precaution we will initially infect any new parasite strains at very low doses.

Administration of parasitic nematode infections: Rats are injected subcutaneously with Strongyloides nematodes at the infective larvae stage. Rats infected with Strongyloides become immune to infection over time. By approximately three - four weeks after infection all nematodes are killed by the rat immune response. We therefore infect new rats every approximately three weeks to maintain the Strongyloides culture.

Collection of faeces: Faeces are collected from the rats and the faecal samples are cultured to grow Strongyloides. The rat faeces are collected by temporarily holding animals in grid-bottomed cages. This is typically carried out twice a week for both general maintenance of Strongyloides in the lab and for the collection of nematodes for experimental analysis. When greater quantities of Strongyloides nematodes are required, for example, if the number of larvae recovered from the faeces is low (recovery of nematode larvae from rats can fluctuate from infection to infection), rat faeces will be collected more than twice a week and up to four times a week. Tubes are provided in each cage to provide relief from the grid-bottom cages.

Collection of Strongyloides nematodes for further analysis: To understand the genetics and biology of Strongyloides nematodes, nematodes will be collected at different stages of their life cycle. Free-living adults, larval stages and eggs will be obtained from cultured rat faeces which have been collected from rats in grid-bottomed cages, as described above.

Parasitic stages of the Strongyloides life cycle will be collected from killed rats. After the rat is killed, parasitic stages can be collected from tissues e.g. gut and lung tissue dissected from the rat. Rats that will be used for dissection of the gut will have their food withheld overnight before they are killed the following morning. This is because it is difficult to collect parasitic nematodes from the gut when it has undigested food inside. Because rats feed predominantly at night, withholding food is required during this time. Withholding food during the day when rats feed at low levels and are more likely to be inactive or sleeping, the gut is less likely to clear undigested food during this time. This would result in fewer parasitic nematodes being recovered from the gut and therefore additional rats would be required.

Typically, Strongyloides infections of rats will be carried out with a single dose of 500 Strongyloides infective larvae. However, we may also use multiple, low level infections (“trickle infections”) which are thought to better represent a natural infection of rats with Strongyloides. For example, this would involve infecting with up to 50 nematodes at any one time for multiple time points (maximum of 10 time points), with at least 24 hours between each injection.

Single housing: When new Strongyloides lines are used, we start by infecting a rat with a single larva. To ensure that the new line is pure we will singly house the infected rat.

Faeces will be collected from the rat to collect the larvae. When there are enough larvae collected from the faeces these will be infected into two rats that will then be housed together i.e. two rats containing the same line of Strongyloides so there is no risk of cross contamination.

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

There are no expected overt adverse effects.

Rats will be injected and some irritation e.g. inflammation or granuloma, may occur at the site of administration of injections.

GA rats with an immune deficiency are more susceptible to opportunistic infections so they will be kept in a biocontained environment and provided with autoclaved water to minimise this risk. When first injecting with new strains of Strongyloides, including GA strains, initially the injection dose of nematodes will be reduced.

Single housing can cause stress in animals but this will be restricted to the period of single housing and will cease when rats are moved to multiple occupancy 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)?

The expected severity is mild for all protocols.

What will happen to animals used in 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 rats in this work is essential. The parasitic nematodes are obligate endoparasites and naturally infect rats and cannot be maintained outside of an animal host. Therefore, there is not currently a non-animal alternative available. The use of rats in this work is essential and the objectives cannot be achieved without the use of rats.

Which non-animal alternatives did you consider for use in this project?
There is no in vitro model available. Culturing of Strongyloides nematodes in vitro can enable some life cycle stages to survive for days-weeks, but under current methods these

nematodes do not develop into the next stage of their life cycle and they do not reproduce.

Parasitic nematodes can therefore not be maintained or studied effectively in this way. This type of parasitic nematode only infects vertebrate hosts and an infection model in an invertebrate species is not possible.

For some of this work the use of animals is essential because we are investigating the response of the host e.g. to understand anthelminthic resistance. There is currently no alternative method available to test this.

We are currently pursuing alternative options such as using organoids to maintain Strongyloides parasites but these models have not been established yet.

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 use well established methods to infect the rat host with the parasitic nematode, using the optimal safe dose of nematodes so that the fewest number of rats are used. This project will use the minimum number of animals to generate the parasitic nematodes necessary to achieve the objectives of the project. These numbers have been calculated based on extensive previous experience. The minimum number of rats required to generate robust and biologically significant data will be used. Experimental designs will be implemented e.g. the use of controls to maximise the robustness of the data generated using the fewest number of animals. Where relevant advice will be sought from a statistician.

Where appropriate genetically altered rats are used which can maintain a nematode infection for longer periods of time. Although a slight harm is caused to the rat because it is genetically altered (n.b. genetically altered rats will be purchased from suppliers and not bred as part of this proposal), the number of rats used is reduced.

From experience, the genetically altered rats such as nude rats which will be used here, have good health and normal behaviour during infection with Strongyloides nematodes.

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

For the molecular and genetic studies we aim to use three replicates for experimental and control groups. Three replicates are sufficient to give repeatable, reliable and scientifically important results. This is based on both experience (this number of replicates has been adequate to generate repeatable and statistical significant results for similar published

work) and based on power calculations.

Where appropriate we will use the NC3RS Experimental Design Assistant for guidance.

Appropriate statistical tests will be used for each data set. An experimental design has been developed for each experiment so that where appropriate. The control group used will depend on the experiment and will either comprise non-infected rats (vs. infected rats) or a comparison between different nematode life cycle stages (e.g. parasitic vs. free-living nematodes). Where appropriate randomisation of rats to experimental/ control groups and blind studies will be carried out. Experiments will be conducted and recorded with the aim of publishing results in accordance 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?

Power calculations and NC3R's Experimental Design Assistant will be used during experimental design where appropriate.

Multiple tissues will be used from the same rats where possible e.g. where collection of parasites from the intestine and collection of other tissues such as the lungs can be coordinated.

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 rats because they are a natural host of the parasitic nematode Strongyloides.

Using the natural host is important because it means that we will study the natural infection process that has evolved.

During infection, no noticeable harm is caused to these rats and eventually the rats become immune to the nematode infection. We will use doses and routes of infection such as subcutaneous injection, that best mimic the natural route of infection and that cause minimal harm to the animals to achieve the scientific objectives.

We will use nude rats and other GA rats with other immune-associated deficiencies.

GA rats will include rats deficient in aspects of the Th2 immune response, the immune response most commonly associated with helminth infections. In these rats the infection lasts for longer, because the rat does not build immunity to the nematode infection.

We use the Strongyloides nematode because as well as being an important parasite of humans and other animals, it is a well-established laboratory model system in rats, to

study parasite infection.

Because methods and protocols are well-established in this system (e.g. infection protocols for the number of larvae to infect with) we know from collective experience of researchers working in the field that this work has a severity level of 'mild' and the chance of adverse effects is very low.

Anthelminthic drugs: Rats may be administered with an authorised anthelminthic drug such as ivermectin and albendazole, and will be administered either subcutaneously or orally including by gavage (AA), at the recommended dose.

Collection of faeces: Rats will be temporarily housed (maximum 17 hours) overnight in grid-bottom cages containing a tube for collection of faeces. Typically, this will be carried out twice a week.

Single housing : When using a new Strongyloides line to assure it is pure and to prevent rats from contamination with a different line of Strongyloides, we will singly house the infected rat. Rats will be single housed a maximum of one time.

Throughout the single housing period rats will be in cages next to each other and they will be able to see each other and communicate vocally.

Collection of rat tissue for further analysis.

Rats will be killed and dissected to harvest tissues including tissues from the gastrointestinal tract, lungs and lymphatic tissue associated with the gastrointestinal tract. DNA, RNA, protein and immune cell samples will be prepared from these tissues and analysed.

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

Adult or juvenile rats are required as a host for maintaining a Strongyloides infection.

Animals must be alive and have a developed immune system in order for a successful infection to take place and to obtain results that are representative of the host-parasite interaction that occurs with an immune response. Rats in particular are used because these are a natural host of Strongyloides parasites and results from these experiments will therefore represent a natural infection and be more informative that an infection in a non-natural host. Strongyloides parasites are also very specialised to a narrow host range and infection in an alternative host organism e.g. an invertebrate would not be suitable or successful.

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

There are no expected overt adverse effects. The expected severity is mild for all protocols. Rats will be injected and some irritation e.g. inflammation or granuloma, may occur at the site of administration of injections. In this case the advice of the NVS will be sought and followed. GA rats with an immune deficiency are more susceptible to opportunistic infections so they will be kept in a biocontained environment and provided with autoclaved water to minimise this risk.

Some irritation at the site of repeated injections (‘trickle infections’) may occur. In this case

the advice of the NVS will be sought and followed. To minimise the risk of irritation at the site of injection there will be a minimum interval time of 24 hours (trickle infections). Rats showing signs of irritation will not receive additional injections at this injection site.

All animals exhibiting overt signs of suffering or (e.g. signs of ill health, pain and distress including, pain and distress including piloerection, hunched posture with reduced locomotion, sunken eyes, marked weight loss, abnormal gait, inactivity or inappetence) will be monitored more frequently and supportive treatment provided such as warming and wet mash. If signs persist for a period of 24 hour rats will be killed by a Schedule 1 method.

Rats that develop diarrhoea or dyspnoea will be killed.

Contamination of uninfected or control rats with parasites from infected rats held in the same facility or room has not occurred previously. To ensure this, uninfected and control rats will be housed separately from rats infected with a parasite. Also, areas where rats are handled will be disinfected between treatments of control and infected animals and person(s) carrying out the procedure will use fresh gloves.

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

PREPARE guidelines will be referred to when planning experiments.

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

We will regularly check the NC3RS website, NC3Rs Experimental Design Assistant and attend 3R- related meetings and events. We also receive monthly updates by email from the NIO and regular emails from the ARLO. I will attend national and international Strongyloides conferences to keep up to date with the latest developments e.g. using organoids to culture Strongyloides.

75. Development and function of neuronal connectivity

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

Neural development, Neurodevelopmental disorders, Synapse, Plasticity, Neuronal activity

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 aim of this project is to understand how connections in the brain form during development, and how these circuits function, both in health and in disorders of brain 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?

We expect this work to lead to a greater understanding of the normal developmental processes that occur during the formation of connections, or 'synapses' between brain cells (neurons) and the circuits they become part of in the developing brain, and how these processes are perturbed in models of neurodevelopmental disorders such as autism spectrum disorder (ASD), intellectual disability, epilepsy and schizophrenia. ASD affects

about 1% of the population and is associated with social communication and behaviour deficits as well as repetitive behaviours and sensory processing issues. Developmental epilepsies are often associated with seizures, which can be very distressing and debilitating. Schizophrenia is a long term, serious mental health condition associated with hallucinations and delusions, as well as other symptoms such as disorganised thinking and behaviour.

There are currently no treatments for ASD that can alter its core symptoms or improve the long-term outcome. Treatment options for other neurodevelopmental disorders are limited and could be significantly improved. To develop and design new therapies, we need to understand the mechanistic causes of these disorders.

This work will significantly increase our understanding of this, will allow both us and others to test candidate drugs, and should pave the way for novel approaches to treatment.

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

We will have an improved understanding of how neural circuits develop and how this process is altered in models for neurodevelopmental disorders. This will result in new publications.

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

In the short term, the increased understanding of how circuits develop and change in models of disorder will benefit researchers working on similar topics, allowing others to build on our results. We will post our data so it is freely available and can also help other researchers. On a medium timescale of 5-10 years, our work will be of significant interest to the pharmaceutical industry and clinicians, and indeed we have established significant collaborations with both these groups. In the longer term beyond this, should we or further work based on our results develop new treatments, these will be used by affected individuals, to the benefit of them, their families and wider society.

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

We will publish this work in high profile journals and disseminate the results in national and international meetings, as well as on social media to maximise reach. In turn, this will promote opportunities for further collaboration and development of new avenues. All manuscripts will be uploaded to the global standard server 'BioRxiv' in order to ensure timely and open availability to the community of our work, and following publication data will be made available as well. Publication will be freely available via open-access.

Species and numbers of animals expected to be used.

- Mice: 20000
- Rats: 450

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 make discoveries about mechanisms that will eventually lead to benefits for people, we need to be able to conduct invasive experiments that are not possible to do in humans, but at the same time it is critical to achieve a balance between relevance to humans and sentience, and we feel that rodents (mice and rats) offer the best compromise. Mice offer the advantage of the availability of transgenic (where genes have been altered) mouse lines, including conditional ones. This allows us to investigate the roles of gene mutation with much greater specificity, where it is possible to turn off or on individual genes in specific types of cells and/or at a particular time during development. Additionally, this means that these manipulations will have much milder, if any, effects on the animal as only a small proportion of cells are affected. Rats allow more sophisticated behavioural tests and tasks, as well as showing responses to drugs that are more similar to humans, and increasingly genetically modified ('transgenic') models are available. Using rodents builds on the wealth of knowledge and research already available, including our own published data, allows use of already-optimised methods/protocols and minimises unnecessary repetition. We will study animals from early development prenatally to adults. This is because to understand development and assess the eventual impact of developmental alterations, we need to follow this process all the way from early stages to adults, so including pregnant adults, eggs, embryos, neonates, juveniles and adults.

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

The majority of animals (80-90%) will undergo genetic alteration with minimal effects on the animal and tissue removal following schedule 1, or terminal anaesthesia (non-recovery) for removal of live tissue.

About 5-10% will only undergo mild procedures such as behavioural tasks or single administration of a drug. About 5-10% will undergo moderate procedures such as surgery under general anaesthesia. The duration and number of procedures will vary a great deal depending on the experiment: given our focus on development this will usually (80-90%) be between 3 days and 3 weeks, with most of the remainder between 3 weeks and 3 months.

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

In general we do not expect any harmful phenotypes from our genetic manipulations. Only 1-2% mice will be bred under the 'moderate severity' breeding protocol, where they can exhibit hyperactive, repetitive or circling behaviours. Animals undergoing surgery or injections are expected to make a rapid and unremarkable recovery.

Rarely, post-operative complications can occur such as infection (<1%) or anaesthetic complications (<1%) but all risks will be minimised wherever possible and no post-operative adverse effects will last longer than 48 hours. For regulated behavioural tests (<2% animals) food restriction to maintain body weight at 85% of freely feeding mice will last for the duration of the experiment, typically 1-2 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 (~80%) of the genetically altered animals we use have no adverse effects, as in

many cases we only affect a small subset of cells in the brain which means that behaviour is not affected and there are no obvious changes. We only anticipate generating 1-2% of animals with a moderate severity phenotype. In addition, a large proportion of our work involves taking tissue for in vitro studies following schedule 1.

Based on our previous work, we expect about 80-90% of animals to be in either sub-threshold or non-recovery categories, 5-10% in the mild category, and 5-10% in the moderate category, with no animals in the severe category.

What will happen to animals used in 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?

In order to understand the fundamental processes underlying the development of synaptic and circuit development of the brain, and how these are disrupted in developmental disorders, we need to be able to manipulate the expression of genes and conduct invasive experiments that are not possible to do in humans. Importantly, we will conduct a significant amount of the proposed work in vitro and ex vivo, predominantly in acute or cultured brain slices and cultured dissociated neurons, which will complement our study.

However, to fully understand mechanisms that underlie normal development and how these are changed in models of brain disorders, we will need to conduct work in vivo as well.

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

Our work will complement ongoing research by our collaborators using non-invasive imaging in patients and will aid in providing a better understanding of how the brain works as well as more detailed information on the structure of the brain. In terms of non-animal alternatives, we also considered using neurons derived from a special kind of human stem cell that has been created from skin or hair cells.

Why were they not suitable?

We collaborate wherever possible with colleagues carrying out non-invasive imaging in humans. However, the resolution (both in space and time) is very poor, and of course it is not possible to do any form of invasive manipulation. In order to understand mechanisms, it is only possible to carry out these experiments in animals. It is possible to use neurons derived from skin or hair cells which have been taken from human controls or patients, transformed into stem cells and grown in a dish, and we have in fact used these in the past. However, this technology is limited in terms of levels of development, lack of (or very altered) structural anatomy, difficulties in generating certain cell types and ensuring physiologically relevant relationships, and very high variability, so the use of animals is 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 based on calculations of requirements for already funded grants, which in turn depends on variability, effect sizes and previous experience with contingency, plus making allowance for further funding in the next 5 years. Most of the numbers are from breeding and maintenance, in order to obtain correct crosses.

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

We used power calculations based on our previous work and similar work in the literature to ensure appropriate numbers of animals to achieve experimental goals, with a careful statistical design and statistical advice. Where possible we have minimised variability. We are also using the NC3Rs Experimental Design Assistant to optimise this process.

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 effective breeding programs to limit the numbers of animals born. Where possible we will use longitudinal measurements of brain activity from the same animals.

This will yield data with great intrinsic scientific value (how neuronal circuits develop) and decrease variability. Where possible we will share tissue with other groups interested in the similar genetic manipulations. We are developing a collaboration (funding applied for) to allow more computer modelling of developmental processes, which may in turn also allow us to minimise 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.

We will do these experiments in rats and mice. We need to use both as mice allow complex manipulation of genes, but rats can perform more complex behaviours and show responses to drugs that are more similar to humans. The methods outlined in the protocols in this application have been carefully chosen to allow us to achieve our objectives while

minimising animal suffering. The vast majority of animals will only undergo a single procedure, and much of the work (80-90% of animals) will be done in fixed tissue or in vitro / ex vivo using tissue obtained after Schedule 1 or terminal anaesthesia of GA animals with no evident phenotype and no other procedures. All animals undergoing surgery will be given analgesics to minimise suffering and the electroporation system we will use has been shown to result in optimum survival and minimal tissue damage. Further, many of our preliminary experiments will be conducted either in cell culture or in live tissue taken from wild type rodents which will enable us to plan experiments and minimise animal usage and suffering. Finally we aim to implement a variant on the standard methods of in vivo imaging that is much less invasive and therefore represents a refinement of technique.

We do not plan to use any protocols categorised as severe.

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

It is critical to achieve a balance between relevance to humans and sentience, and we feel that mice and rats offer the best compromise. The regions of the brain such as the cortex, that are known to be important for neurodevelopmental disorders, are reflected in mice and rats, and choice of model can be validated with relevant behavioural phenotyping (which, although caveats apply, are more relevant than, for example, in invertebrate species, and impossible in purely in vitro approaches). For example, zebrafish do not have a layered cortex - a fundamental feature of rodents and humans - and their behavioural repertoire is limited. Invertebrates such as Drosophila do not have a cortex at all, and an even more limited behavioural repertoire, especially regarding complex higher behaviours relevant to neurodevelopmental disorders. Further, mice are ideal due to the number of transgenic mice available including disease-relevant mutations and reporter lines, and increasingly transgenic rats are becoming available as well. Although currently there is limited availability of transgenic rats, rats have a wider repertoire of behavioural tests available and show better pharmacological alignment with humans so increasingly will be the model of choice. The availability of inducible and conditional mouse lines allows specific targeting of developmental stages and cell types/circuits, which also minimises adverse effects. Additionally, in collaboration with molecular biologists, we can reduce or overexpress candidate molecules and examine their effect on the development of connectivity. Working with rats and mice also builds on the wealth of knowledge and research already available and minimises unnecessary repetition. Finally, while we will focus a great deal of our work on more immature life stages, as we seek to understand developmental processes, in order to assess the impact on adults we will need to also study adult animals.

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

To minimise animal suffering we will continually employ refinements to our techniques and procedures. For example, when the technology is ready we aim to switch to ultrafine optical fibres for in vivo imaging, which are far less invasive compared with current techniques. We will also continuously refine the procedure for behavioural training, by using computer programmes to enable more efficient training.

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

I will follow the following published guidelines:

The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments)
NC3Rs-funded video tutorials on the Research Animal Training website (https://researchanimaltraining.com/article-categories/aseptic-technique/)
LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery
PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am subscribed to regular email newsletters from NC3Rs (as well as the EDA). I also liaise closely with other members of my department, where a colleague is a member of a working group at NC3Rs, and researchers at other institutions to discuss advances. At my institution, the NACWO and NVS will also advise on these advances.

76. Modelling Mixed Vascular-Alzheimer's Dementia (MVAD) in mice

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Mixed dementia, Vascular dementia, Dementia, Platelets, Cerebral blood flow

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?

Dementia is the progressive loss of function of the brain. It is associated with the damage of the brain tissue and increasing evidence suggests that the loss of function of the brain blood vessels plays a key role in dementia. There are currently no cures for dementia.

Therefore, research into the mechanisms of dementia is extremely important for healthcare. In our previous work, we discovered that platelets (small circulating cells responsible for blood clotting) deposit in the brain blood vessels of mice modelling dementia. These data correspond to observations from autopsies of human patients with dementia, which highlighted the damage of brain small blood vessels caused by platelet accumulation (also known as “brain microthrombosis”). In this project, we aim to utilise a well-established mouse model of dementia to 1) confirm and quantify the association of platelet deposition and brain vessel damage with the progression of dementia; and 2) test whether platelet-inhibiting drugs can help to protect brain blood vessel health and potentially fight dementia.

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 no cure for dementia and an increasing portion of society is affected by this disease because of the ageing of the population. Investigating dementia is therefore a critical goal of modern biomedicine. Here, we propose an innovative approach that could significantly improve our understanding of dementia and indicate new avenues for its treatment by targeting the health of brain blood vessels and potentially slowing down or reversing the progression of the disease.

What outputs do you think you will see at the end of this project?

This project will:

- provide novel information regarding the role of platelets and blood clotting in dementia progression.
- determine whether antiplatelet drugs such as Aspirin or Ticagrelor can be beneficial for the protection against dementia progression.

Who or what will benefit from these outputs, and how?

In the short term, academic scientists and pharmaceutical companies will benefit from the identification of new mechanisms of disease progression and novel drug candidates for the treatment of dementia. In the longer term, following validation of our discoveries in humans, clinicians and patients will benefit from our discoveries because of the introduction of brain blood vessel-protecting drugs in the routine treatments against dementia.

How will you look to maximise the outputs of this work?

To maximise the output of this project, the results will be:
Presented at national and international research conferences, such as the International Society of Thrombosis and Haemostasis (ISTH) conference, and the European Vascular Biology Organisation (EVBO) conference.

Published in world-leading research journals; to remove barriers in the fruition of our research, only Gold or Green Open Access publication modalities will be chosen (e.g. JISC membership and agreements with selected publishers such as Elsevier, SAGE, Springer, and Taylor and Francis).

Communicated through press releases of the university and in a dedicated section on the hosting laboratory website.

Shared with the entire research community and the general public via the Hydra repository; this will be particularly important for the disclosure of negative data and raw data, which are normally difficult to share using traditional tools such as conferences and research journals but are extremely important to avoid unnecessary research duplication.

The raw data shared on Hydra will be in a variety of easy- access formats: text files (e.g. Word), statistical analysis files (e.g. Excel), raw output files (e.g. ASCII files) and image files (e.g. JPEG or TIFF).

Species and numbers of animals expected to be used.

- Mice: 20 wild type C57BL6/J (10 per sex) and 280 3xTG-AD mice (140 per sex) for experiments, while 40 3xTG-AD mice will be required for the maintenance of breeding

colonies. The total number of animals in 5 years is 340 mice in total.

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 utilise mice that express three gene mutations inducing dementia. We chose a strain of mice called 3xTG-AD, which develop symptoms of dementia between the ages of 6 and 12 months. 3xTG- AD mice display brain tissue degeneration and impaired cognitive abilities, but do not show signs of other adverse effects or reduced survival rates compared to control mice. This is a well-established animal model to study dementia. We will also use wild-type mice as negative controls (strain: C57BL6/J), in which brain degeneration and cognitive decline do not occur under the age of 12 months.

Typically, what will be done to an animal used in your project?

3xTG-AD mice have been characterised in detail and no reduction of life span or welfare was identified compared to normal mice. We are planning to maintain 3xTG-AD mice up to the age of 12 months, at which stage brain plaques have appeared and the cognitive ability is reduced, but no pain, suffering or distress has been documented. The drugs that we are testing in this project (i.e. Aspirin and Ticagrelor) have already been abundantly tested in mice without any sign of toxicity or other adverse effect.

Nonetheless, we will regularly monitor the health of the mice throughout the project using daily checks for sign of toxicity, illness or discomfort. The novelty of our project is to test the effect of these drugs on dementia progression. At the end of the project, the mice will be humanely sacrificed under non- recovery anaesthesia to harvest and preserve brain tissues that will be used to assess the effect of the tested drugs on brain health.

What are the expected impacts and/or adverse effects for the animals during your project?

3xTG-AD mice have impaired cognitive abilities, but do not show signs of other adverse effects or reduced survival rates compared to control mice. The proposed drug treatments have been widely tested before and do not have any adverse effects. Nonetheless, we will regularly monitor the health of the mice throughout the project using daily checks for signs of toxicity, illness or discomfort. The following signs of bad health and discomfort will be monitored: 1) Appearance (e.g. hunched posture, sunken eyes, staring coat), 2) Behaviour (e.g. inactivity, restlessness, increased aggression on handling, unresponsiveness or segregation from other animals, reduced feeding); 3) Weight loss; 4) Clinical signs (e.g. eye or nose discharges, diarrhoea, dehydration).

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)?

Wild-type control mice: mild severity from the drug treatment

3xTG-AD mice: mild severity from the dementia-inducing mutations with or without the drug treatment.

What will happen to animals used in 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?

These experiments need to be performed in mice because only experiments with live animals allow testing of the complex pathophysiological phenomena that we are interested in. No in vitro experimental setup can model and investigate multiple complex phenomena such as gradual brain deposition of plaques, deterioration of brain blood supply, and degradation of brain tissue mirroring the human condition.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives were utilised to generate data that justify the progression to animal experiments. We utilised human and mouse platelets and vascular endothelial cells treated in vitro with amyloid peptides (i.e. the main component of brain plaques) to confirm the effect of plaque material on blood clotting and brain blood vessel function. In addition, we studied human and mouse platelets in vitro and found that they release amyloid peptides, which suggests the possibility that these cells participate in the deposition of brain plaques during dementia progression, rather than simply responding to it.

Collectively, these observations need to be tested in a living organism with anatomy and physiology similar to humans to be confirmed.

Why were they not suitable?

In vitro experiments cannot reproduce the biochemical, cellular and physiological complexity of dementia onset and progression occurring in a 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?

Yes, we utilised previous experiments performed in our laboratories and the resulting data to estimate the correct animal numbers required to obtain statistically robust results.
What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We planned to utilise tissues from the same mouse for a variety of experiments, which will reduce considerably the number of animals required 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?

Statistical tools (e.g. power calculations using previous data) were utilised to calculate the number of mice required for these experiments. Statistical analyses of the results will be utilised during the project to inform regarding the requirement for further experiments.

Where possible, we will use fewer mice than originally planned when the statistical significance of the results is reached.

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 utilise wild type and 3xTG-AD mice. 3xTG-AD mice are a well-established model of dementia that we have shown to display both markers of mixed vascular Alzheimer's dementia, which are plaque deposition and blood vessel impairment in the brain. As indicated by scientific literature, these animals do not experience any severe or moderate discomfort in their 12 months of age and the drug treatments chosen do not cause pain, discomfort or health risk.

Drug treatments (well-tested and non-toxic) and experimental procedures (non-recovery perfusion fixation) were chosen to minimise animal suffering.

Why can't you use animals that are less sentient?

The choice of adult mice as an experimental model is necessary to collect reliable information on dementia onset and progression. Less sentient living organisms of life stages will not allow the investigation of the events and mechanisms that we are interested in.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mouse health will be regularly monitored during the project and signs of bad health or discomfort following standard internal procedures and in collaboration with NTCO and NVS. Clear endpoints were set that will trigger humane culling of the mice if necessary.

The standard of animal maintenance will be regularly updated and improved throughout the life of the project in collaboration with the Biomedical Research Facilities staff of our

establishment.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the PREPARE and IMPROVE guidelines. In addition, we will constantly update our understanding of experimental animal welfare through NC3R, LASA and FELASA webpages and documents.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through scientific literature, including NC3R, LASA and FELASA webpages and documents.

77. The health consequences of developmental exposure to the real-life environmental chemical mixtures

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

Metabolic syndrome, Cardiovascular disease, Infertility, Environmental chemicals, Sex differences

Animal types	Life stages
Sheep	pregnant, 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 assess and compare the effects of developmental exposure to real-life Environmental Chemical (EC) mixtures (e.g. mixtures of flame retardants, plastics, pesticides, personal health products, drugs) on health outcomes in males and female offspring.

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 healthcare faces several immediate, significant and interlinked challenges.

Specifically, there has been a dramatic increase in metabolic diseases, including type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD) and obesity, collectively referred to as metabolic syndrome (MetS), and declines in cardiovascular health and fertility, that may or may not be a consequence of metabolic disease.

Although many factors are likely to contribute to these health conditions, there is increasing evidence that they are associated with exposure to environmental factors, most notably chemicals which if they enter our bodies can disrupt normal function.

The situation is made more complex, as it is becoming increasingly understood that disease risk in response to chemical exposure is also influenced by genetic factors, and may be transmitted across generations. Risk is also influenced by sex, thus understanding the risk posed by exposure to chemicals on males and females is crucial to understand.

The actual health risks, of exposure to the complex mixture of chemicals that exists within our environment, remain poorly understood as many studies have been limited to short term studies where individuals are exposed to high concentrations of single chemicals. Furthermore, many studies have only been conducted in animal models that have a different developmental pattern to humans e.g. rodents and have often only been undertaken in a single sex. This study will extend our existing body of work in this area by examining the health effects of real-life EC exposure across multiple life stages, in a translationally relevant animal model.

What outputs do you think you will see at the end of this project?

The primary outputs of the work will be information that will detail the effects of exposure to a mixture of the environmental chemicals (ECs) on a variety of body systems, such as the reproductive and cardiovascular systems and on metabolic health. In doing so, we will also get information as to how physiological processes are changed following mixed EC exposure and if the effects of EC exposure are different in males and females. These outputs may be used to inform the development of regulations and policy to help protect human, animal and environmental health. An additional output will be the extension of a store of tissue samples (made available to researchers interested in the effects of developmental exposure to ECs).

Who or what will benefit from these outputs, and how?

A wide variety of stakeholders will benefit from the outputs including 1) Policy making bodies; regulatory and monitoring agencies (e.g. Water companies, National Environmental Protection Agencies), so as to inform activities to safeguard the general public and wild/companion/domestic animal populations. 2) Clinicians and researchers (human and veterinary), due to the potential role(s) of EC exposure with regard to changing trends in public health and the mechanisms that underlie such changes in the patterns of disease risk. 3) Associations responsible for animal welfare and environmental protection (e.g. National Farmers Union, Compassion in World farming), due to the implications for farming practice and the care of managed and wild animals. 4) Companies associated with wastewater treatment, as our work may inform which chemicals need to be targeted or removed from the solid waste (biosolids) that they generate, to improve environmental safety. 5) Students and researchers will benefit from data generated by this project, which may inform their areas of research. Students will also gain from training in environmental toxicology, chemical analysis and physiology and veterinary/undergraduate

students may have opportunities to become involved with in vivo large animal experiments. 6) Our work will also be of interest to the general public, due to the swell of public opinion regarding the contamination of waterways with human wastewater, waste management, ECs and how use of chemicals may ultimately affect our health and the health of the animals and the environment around us.

How will you look to maximise the outputs of this work?

This project will capitalise on a unique animal model and builds on an existing international collaborative project to investigate the mechanisms by which a mother's exposure to a real-life cocktail of chemicals during pregnancy can affect the long-term metabolic, cardiovascular and reproductive health of her offspring and that of subsequent generations. The principal outputs will be peer reviewed scientific papers in open access publications and open data that will be made freely available to other researchers upon request. In addition, we will curate a tissue biobank that has been established and is available to researchers interested in the effects of developmental exposure to ECs.

Species and numbers of animals expected to be used.

- Sheep: 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.

The proposed work will be conducted using sheep as an animal model for humans and other animal species. The nature of the work requires use of a whole animal model and cannot be accomplished using laboratory-based systems, computer simulations, or mathematical models. This is because these alternate study systems do not, as yet allow assessment of the interactions and age-related changes that occur between complex physiological systems i.e. the metabolic, cardiovascular and reproductive systems, when they face complex challenges such as to a real-life cocktail of environmental chemicals (ECs). Sheep have been chosen as the most appropriate animal model for a variety of reasons.

Importantly, sheep exhibit numerous features that make them more appropriate for comparative studies with humans compared to other species (e.g. rodents). Sheep are ideal for reproductive/developmental studies, as they have a long pregnancy, are born in an advanced state of development and have an extended pre and peripubertal period (similar to humans). Given their relatively large body size, it is possible to collect repeated blood samples from sheep; and at postmortem, their large organ size means we are able to collect multiple samples for subsequent tissue and anatomical analyses, increasing what we can learn. Relative to rodents, sheep brains are more comparable to human brains with regards to size, blood flow, how cerebospinal fluid moves within the brain. The timescale over which the sheep brain develops before and after birth and how it is organised is also more similar between sheep and humans compared to rodents. The body size of a sheep is also more comparable to a human, than that of a rodent, and this is beneficial with regard to the assessment of the effects of ECs on cardiovascular health. Sheep and humans also use the same biochemical pathway, which differs from that used

by rodents, when producing some steroid hormones. Due to the role of sheep in comparative studies and their agricultural importance, extensive information on sheep physiology and behaviour is available from the literature making the interpretation of findings possible.

The patterns of EC exposure to be studied may cover a number of important periods of development, such as during pregnancy and/or before and during the pubertal transition. This is important, as we know that many adult-onset diseases are programmed during such important periods of development, and in real-life, EC exposure may occur across multiple life stages or whole of life, for humans and animals.

Typically, what will be done to an animal used in your project?

The 'experimental manipulation' will be the grazing of pregnant sheep on pasture treated with biosolids. Biosolids are a by-product of wastewater treatment. The use of biosolids as an agricultural fertiliser is accepted husbandry practice and the application of biosolids and subsequent use of biosolids treated pasture will follow all government guidelines and regulations. The nitrogen content of applied biosolids is matched to that of traditional inorganic fertiliser which will be used on control pastures, so that there will be no difference in the nutritive value of the pasture to the grazed animals. For mating of animals, ewes will be synchronised as per normal husbandry procedures. While this may require a short-term restraint and sheep may experience some discomfort during the procedure, they will not experience any lasting harm. Once pregnant, to assess any effects of the environmental chemicals present in biosolids that transfers to the mothers and which could indirectly affect the offspring, mothers may be blood sampled at up to two timepoints, typically days 60 and 90 of pregnancy. This would require very short-term restraint and while animals may experience very mild pain or discomfort during the collection of samples they will not experience any lasting harm. The offspring of mothers exposed to biosolids will be maintained up until, and into adulthood. The offspring will be studied over time using well characterised methodologies such as physiological challenges, collection of blood samples, measurements of body size, non-invasive assessment of the activity of systems that regulate heart function (heart rate variability), blood pressure, and assessment of heart structure and functionality using ultrasound (echocardiography). Ovarian function may be assessed using trans-rectal ultrasound (female only) and semen may be collected from the males. These procedures would typically require short term restraint, and while animals may experience very mild pain or discomfort they will not experience any lasting harm from these assessments. The above assessments will not all be conducted on 300 animals, within each experiment, groups of animals (typically n=8-12, based on power calculations using data from our earlier studies) will be used to assess effects of ECs on different physiological processes and functions. A subset of animals identified for body size measurements (non-invasive), may have these measurements conducted monthly over their lifetime. Animals in which heart function is assessed, would normally be studied over a 17 day period in which blood pressure would be measured 3 times (over 5-15 minutes) for 15 consecutive days, heart rate variability would be assessed on one day and echocardiography performed on one day (all non invasive). Animals that receive an endocrine assessment, could require blood samples (up to 10, ~4-6ml samples) to be collected over a period of 1 to 2 hours, after administration of a hormone or sugar solution. Animals in which reproductive hormones were to be assessed could require blood samples (up to 55, ~4-6ml samples) to be collected over a period of 3 weeks. At defined timepoints, cohorts of animals will be euthanised and tissue samples collected.

What are the expected impacts and/or adverse effects for the animals during your

project?

Grazing animals on biosolids-treated pasture is an accepted husbandry practice (biosolids as a fertiliser is used in agricultural land across the UK and worldwide with no monitoring required) and is not expected to cause any adverse effects beyond subtle effects that are the subject of study in this project. Our use of biosolids will adhere strictly to the UK Government Sewage Sludge (Use in Agriculture) Directive which includes a 3 week "no graze " period following spreading. As the grazing of sheep on biosolids treated pasture is a “recognised husbandry practice” it is not regulated under ASPA.

The majority of the measures that will be assessed over time in animals (e.g. Blood pressure, heart rate variability, ECG, body size) are non-invasive although they may require temporary restraint (2- 15mins). Trans-rectal ultrasound (female only) would also require animals to be restrained for a short period of time but is standard veterinary practice. While it may cause mild discomfort transrectal ultrasound should not result in lasting harm. Semen collection would be by natural mount and therefore is not expected to have any impact or adverse effects on the animals involved.

Where blood samples are to be collected, animals will be subject to restraint and samples collected from a peripheral vein using a vacutainer or a syringe, these could cause mild discomfort and pain but will be done by trained and experienced personnel to minimise these effects. The volume of individual samples will typically be 4-6 mls and the total volume of blood collected at any one time or over a 28-day period will be a small fraction (<10%) of total blood volume and thus will pose no risk to the animals. The incidence of local or systemic infection following blood sampling is typically very low (<0.05%).

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% of the procedures that would be conducted on the mothers (F0) would be mild.
F0 males will not be on procedure.
90% of the procedures that would be conducted on the F1 generation are non-invasive and thus would be subthreshold, 10% will be mild.

What will happen to animals used in this project?

- Killed
- Kept alive at the establishment for non-regulated purposes or possible reuse

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 look at the interrelated effects of exposure to an environmental chemical mixture, and how any effects change over age and across generations, with regard to the regulation of body systems including metabolism, cardiovascular function and

reproduction. As there is cross communication between these systems, at multiple levels e.g. via endocrine and neural signalling, to date, these studies cannot be accomplished using laboratory-based systems, computer simulations, or mathematical models. In addition, laboratory-based systems do not show the patterns of change that are seen in physiological systems and the cross talk between such systems in animals as they age.

As such the use of a whole animal model is required to address the aims of this project.

Which non-animal alternatives did you consider for use in this project?

Cell cultures can be used to assess some aspects of environmental chemical exposure and as part of the tissue bank we have created from our existing project, we have generated a bank of cells that can be grown in the laboratory and which can be used to look at some effects of environmental chemical exposure. Such cell culture systems, however, typically allow questions to be addressed that relate to single cells or single systems and thus are not suitable to address the aims of the work to be conducted under this licence as they are not integrated with other physiological systems.

Why were they not suitable?

Cell culture systems are currently unable to allow assessment of the interactions and changes that occur between complex physiological systems over time and/or between generations 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?

Group sizes were defined following advice from a biostatistician, during the design stages of the submitted grant applications that will fund the work to be conducted under this licence, and based on the amount of variation that naturally occurs in the traits to be looked at and an estimate, based on previously published studies, of the size of any effects of treatment. It also takes into account the potential loss of animals due to other health reasons. In all cases the group sizes chosen are the minimum required but are sufficient for the results of the work to be statistically valid.

The proposed group sizes were also considered as part of the grant review process.

Not all animals to be covered by this licence will undergo all physiological tests, as this is influenced by the specific experiment and the variability in the trait of interest.

Allocation to optional steps will be randomised but controlled for paternal genetics and to ensure that no animal is disproportionally represented in tests.

To reduce the variability that could arise as a result of differences in paternal genetics, the ewes will be blocked (weight and body condition) and randomly allocated to experimental

groups and a limited number of rams will be used to generate the offspring of the Biosolids exposed/control ewes. Rams will be balanced across the treatment and control groups (through use of artificial insemination) and ram will be included in all analyses.

All experiments will be conducted so as to be able to publish the results according to the ARRIVE guidelines and NCRs will be used for guidance with regard to best practice www.nc3rs.org.uk

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We consulted with a biostatistician who conducted power analyses to determine the minimum group sizes required for the generation of valid statistically robust conclusions within the experimental design phase.

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 experiment, we will collect tissue samples for inclusion in the tissue banks (frozen and fixed) that we have established and made available to researchers with an interest in the effects of developmental EC exposure.

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.

Apart from grazing on pasture treated with Biosolids, which is normal agricultural practice, the F0 ewes will only be blood sampled (twice maximum > 30 days apart).

They will be allowed to raise their lambs according to normal husbandry practice and may be returned to stock. As such, they will be subject to only minor discomfort and no lasting harm.

The main focus of the work covered by this licence will be the patterns of physiological function in the F1 generation. As we wish to study normal function in these animals, the majority of work will be non- invasive, and sub threshold. The collection of blood samples will be required for the assessment of circulating hormones and metabolites, in the majority of cases the factors to be studied will be those produced naturally e.g. changes in steroid hormones as animals pass through puberty. However, it may be that factors are studied after routine physiological challenges e.g. administration of glucose or a hormone challenge test e.g. GnRH challenge. In these situations, the substance administered would not be expected to exceed the normal range. None of the investigative work proposed is expected to result in any lasting harm to the animals involved.

Why can’t you use animals that are less sentient?

The translational relevance of the work to humans means that it requires to be completed with a mammalian species. As the work is specifically interested in changes in physiological systems with age, the study subjects will be followed from birth until adulthood.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be studied into adulthood, but studies will not follow animals into 'old age'.

Animals will typically be maintained according to normal agricultural practice/industry standards and some animals will be returned back to stock. Given the nature of the procedures/steps within procedures, no special management is anticipated to be required as welfare costs are not anticipated.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The National Centre for the Replacement, Refinement and Reduction of animals in research website is readily checked. The resources available, such as literature on experimental design and ARRIVE guidelines are reviewed and incorporated into experiments to maintain the most refined procedures are conducted.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The scientific literature is continually reviewed, and veterinarians consulted with regard to alternate study methods to ensure that procedures limit potential for adverse effects. In addition, we will keep abreast of local guidelines developed by our local AWERB and seek their advice where appropriate. To prevent duplication of experimentation and facilitate sharing of tissue samples and data, scientific conferences are attended, and discussions held with colleagues.

78. Understanding unusual cell cycle and motor proteins during cell division in malaria parasite

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Malaria, Cell Division, Motor proteins, Mice, Mosquitoes

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 project's 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 better understand the unusual cell cycle and motor proteins that are found in the malaria parasite. This will give us insight into parasite cell proliferation which is relevant to malarial disease and its transmission. By better understanding how parasite cells divide and multiply, we will be able to identify better intervention targets to treat 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?

The malaria parasite is the most deadly of all diseases that can be spread by mosquitoes, killing 608,000 and infecting 249 million cases worldwide in 2022. With global warming, the need to curtail malaria parasite proliferation and transmission is becoming very important. Understanding the mechanics and the fundamental knowledge of how the parasites proliferate is crucial for any interventional approaches to develop.

What outputs do you think you will see at the end of this project?

We are interested in the proteins that help the malaria parasite multiply and invade a host. The way that the parasite divides is different to how human cells divide and this remains largely unknown. From this project, we will gain knowledge of where the proteins are produced in the cell, how they behave and work, and how they are essential for parasite cell division. This knowledge may help in developing new drugs or vaccines to intervene with their multiplication, thereby controlling the disease. Our findings may also be applied to other similar parasitic diseases.

Most outputs will be published in the form of open access publications in journals and also shared during various conferences, as we have done previously. Outputs will also be shared through our media office to inform the general public of the impact of the work. Any proteins that we find to be important in this project may serve as targets for drug or vaccine development which may in the long- term either be patented through our organisation or shared with the malaria research community. This knowledge will be beneficial not only for the malaria community but also, more widely, for drug development by pharmaceutical companies or other research organisations like the Wellcome Trust. In addition, it will aid in skill development and future collaboration within the scientific community.

Who or what will benefit from these outputs, and how?

The specific benefits of our program of work are as follows: Understanding how the parasite cell works during cell growth and division.

In the short term (2-5 years), we will publish about the unusual mechanism of cell multiplication in the malaria parasite through peer reviewed journals and also through communication at various international and national meetings. This knowledge will be used by scientists in the field of fundamental biology of cell growth and division. This will also foster future collaborations in the subject not only with malaria biologists but biologists working on cell growth in various organisms, like we have recently started to collaborate with various groups **who are expert in cancer and other abnormal cell division**. We will also generate data which will be a resource for the malaria community. It will benefit not only the malaria community but knowledge will be transferable to people working with other organisms like human, flies and yeast to name few.

Medium and Long term benefits
Studying the parasite whilst it grows and the process of parasite multiplication in both human/mice host and mosquitoes will allow us to generate new knowledge and resources. The parasite lines generated will serve as a resource for future studies such as drug discovery programmes and will benefit the wider malaria community.
Since the proteins selected in this project are also ideal candidates to target in cancer, the fundamental biology obtained during the study might also help to understand how abnormal cell multiplication in cancer cells works.

The project also is of benefit to evolutionary cell biologists who are interested in understanding the origin of life and how the cell multiplication takes place in single celled organisms like the malaria parasite and what genes are similar or different in other organisms like human or yeast.

How will you look to maximise the outputs of this work?

As mentioned above the important collaborations, various resources produced previously and published recently and during the course of this project, and publishing our knowledge

in open access journals and also depositing the data obtained through genomics sort of experiment in data bases will maximise the outputs. In addition, the information will be presented in various conferences related to subject.

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.

In this project, we will use the mouse malaria parasite model (***Plasmodium berghei***) that infects mice and mosquitoes. It will be propagated in mice and then used to study both its function in mouse red blood cells and also in the mosquito. It is a powerful model that is safe for humans to work with as there is no risk of infection.

The model allows us to study the entire complex life cycle of malaria parasites which is extremely difficult to do safely with human malaria parasites.

The animals used in this project will be mice and will be infected with mouse malaria parasites. Mice will be used to grow and maintain the malaria parasite. Mice will also be used for breeding and parasite infection in mosquitoes where the mosquitoes are fed on naïve mice blood to obtain mosquito eggs or infected mice blood to grow the parasite within the mosquito host. This will allow us to obtain female mosquitoes for parasite infection and for studying the parasite development in the mosquito host.
Typically, animals will experience only brief, slight discomfort and no lasting harm, from administration of substances by injection of parasites using standard routes (intraperitoneal, intravenous, subcutaneous); most experiments will involve intraperitoneal injection. Some mice will be provided with antimalarial drugs in the drinking water to avoid further injection. Animals will experience slight discomfort from blood sampling to generate a blood smear to check for parasite load.

Typically, what will be done to an animal used in your project?

Typically, a mouse will be injected with some parasites and left for up to 5 days for the parasites to increase in number, being monitored every day for the number of parasites and the health of the mouse. Sometimes, anti-malarial drugs will be used; these will to allow us to select for specific, genetically altered parasites.

When the parasite number has reached a sufficient level the mouse will be anaesthetised, blood collected for parasite analysis, and humanely killed by Schedule 1 method.

Depending on experimental need, sometimes the mice will be anaesthetised for uninfected mosquitoes to feed on the infected mouse’s blood, which may carry different control or genetically altered strains of parasites. The mouse will then either be killed and the blood collected, or it will be allowed to recover to see if the parasite has infected the mouse. Following the mosquito feed, mice will experience slight harm because of the mosquito bite.

What are the expected impacts and/or adverse effects for the animals during your project?

- No adverse effects, exceeding the mild to moderate severity limit, are expected in this project as most infected mice are kept for 4-5 days, however there is an increased chance of malaria related symptoms when they are kept longer for high parasite load as the parasitemia reaches 30%. Some of the possible adverse effects then include:
- Transient discomfort from needle insertion (100% likely incidence).
- The long term effect of parasitemia (parasite load in mouse blood) (100% likely incidence).
- Stress due to restraint and transient discomfort from needle insertion and blood collection (100% likely incidence).
- Bruising / haemorrhage/ haematoma at the collection site (<5% likely incidence). Thrombosis (clotting) / phlebitis (inflammation of the vein) (<5% likely incidence) Some animals may experience anaemia when the parasite load is higher than 10% (<5% likely incidence)
- Bruising at the mosquito bite site during parasite transmission from infected mosquito to naive mice(<5% likely incidence)
- Animals may experience sluggishness, piloerection when the parasite load approaches 30% (<5% likely incidence)

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)?

95% of mice will experience only mild severity as most mice are only kept for 4-5 days after infection and the infected blood is collected at 5-10 % parasite load.
Only 5 % mice may experience moderate severity. This is typically associated with a higher parasite load, ~20%-30% (which may result in e.g. lethargy, hunched posture).

What will happen to animals used in 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 what controls parasite multiplication in the two hosts that the parasite infects (human and mosquito) it is required to use the model that can give fundamental understanding of the full parasite life cycle. Any work on the human form of malaria (*P. falciparum*) is mainly limited to the non- sexual stages of parasite development and it is harder to study in mosquitoes with the ease and robustness that can be obtained with the mouse malaria parasite *P. berghei*. *P. berghei* provides a robust, genetically tractable model and still permits understanding of the human form of malaria (*P. falciparum*), as it shows many similar clinical symptoms, such as cerebral malaria. Many of the genes are

very conserved between two species and *P. berghei* is used as part of a drug discovery programme for *P. falciparum*. By using the *P. berghei* strain of malaria, we can study how genes/proteins work throughout the full parasite life cycle in the mouse and mosquito hosts. Moreover, there is no need for any containment for this purpose unlike human parasites.

Which non-animal alternatives did you consider for use in this project?

We have considered cell culture techniques and in silico approaches.

Why were they not suitable?

While cell culture can be used for the analysis of some of the non-sexual stages, we cannot adequately model the whole life cycle of the parasite by cell culture. To get a full picture of a gene of interest in its in vivo situation, we need to observe it through the whole life cycle. We also use in silico approaches such as Alphafold modelling where we can, but only limited information can be obtained from these approaches. Any information produced in silico ideally should be confirmed in vivo to ensure it is correct. We also use computer modelling like Alphafold to understand the structure of protein molecules before we genetically alter the genes as well to predict the protein interaction so that less molecules are studied 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?

We have estimated the number of mice needed based on past papers and experience in the field. We have over 15 years' experience with many of the techniques we use and have also spoken to other scientists and NC3Rs managers in the field about how much parasite material would be needed for specific experiments. This has allowed us to calculate an estimate of the number of mice needed to obtain sufficient parasite material per experiment. For some experiments, we need up to 10 mice to get enough parasites to yield optimum and rigorous quality results. We have also used our annual returns as a guide to help us estimate the number of mice needed to complete our project. Based on technological advances, we envisage to reduce the number of mice without compromising the quality of results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In the previous project, we estimated that we would need about 8000 mice for our work. Now the protocols we use have been refined with advancements of protein analysis and gene targeting, we can for example use the blood from one mouse for analysing multiple stages of a parasite during functional analysis. This gives power to the work and so we have reduced the estimate of mice needed to 6000 over the length of the licence.
We have seen a group size of 5 mice gives us the ability to accurately analyse the material

and draw valid and rigorous scientific conclusions. This follows the NC3Rs Experimental Design Assistant. We will also make arrangements for conducted studies to be published according to ARRIVE guidelines as we have demonstrated in many publications recently. We also use randomisation and blinding in the experiments to give power to experiments and reduce mice number. In many experiments, a single control with many experimental variables will decrease the number of animals used for control overall.

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, for imaging we can use one infected mouse to get data for multiple stages of parasite multiplication both in mice and mosquitoes rather that one mouse for each stage. We also try to use a small amount of parasite infected blood from the tail vein of the mouse for live cell imaging of parasite stages without culling the mice, hence a number of time points can be obtained from the same mouse which gives power to the experiment and reduces the number of animals used.

The use of the drug phenylhydrazine increases the amount of new red blood cells (reticulocytes) which the malaria parasite prefers to invade thus increasing the parasite infection. This helps in increasing the amount of parasites we can collect per mouse and so decreasing the amount of mice needed per experiment or increasing the number of techniques we can do with the parasites collected from one mouse.

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 malaria parasite model is one of the only models that allows the study of the complete life cycle of the malaria parasite in the laboratory in a living animal.

A minimum number of procedures will be employed which mainly involves injecting the parasites, blood sampling or drug administration in conscious animals by drinking water or injection to minimise any stress to the rodents.

Administering the agents via drinking water (95% of the time) is preferred as it is less invasive but in rare cases injection may be necessary as this is a better method of delivering a set amount of anti- malarial agent.

We will ensure an appropriate volume of parasite is administered to avoid ill health in the mice. Also, mice are monitored daily and kept for a maximum of 4-5 days to ensure minimal suffering due to ill health resulting from parasite number.

Why can’t you use animals that are less sentient?

While malaria is a parasite which can be found in many animals, the mouse model uses the least sentient animal that can give us sufficient blood/parasite numbers to allow us to get rigorous knowledge about the biology of the parasite. It is a powerful model to study parasite multiplication in vivo in both the mouse and mosquito host which is not possible with the human parasite. The mice need to be of sufficient age and size (usually we use mice 8-10 weeks old) so we can collect a sufficient amount of blood/parasite number for any study. The animals will be terminally anesthetised before cardiac puncture is used to collect the blood. The mouse is the only host to the parasite (*P. berghei*) we use.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

By taking blood smears from mice on a regular basis we can control the number of parasites per mouse which will typically be in a 24hrs period and no more than 15% in 28 day period. 95% of animals in the project will be kept for 4-5 days and then used for collection of infected blood when it has a parasite load of 10%. All animals will be checked for adverse symptoms on a daily basis. For 5% of animals the parasite load will be allowed to increase to a maximum of 30% to allow us to get sufficient material in an experiment and minimise the number of mice used. Once the parasite load has passed 10%, daily assessments of parasitemia will be carried out with about 4 blood smears being needed before a level of 30% is achieved and termination occurs. By doing this, any suffering an animal may experience can be kept to a minimum. Most infected animals in the project will only suffer mild severity.
If we detect a parasite load of greater than 30% (this is where 30% of the red blood cells in an animal contain a parasite) or if any animals display any of the symptoms of adverse effects like reduced motility, piloerection as discussed for each protocol, the experiment will be stopped and the rodent killed immediately. We only expect about 5% of total animals used to experience a parasite load of 20- 30%
We follow frequent monitoring at least twice a day and also during weekends. During the weekend if any mouse shows any adverse effects then staff including BSU staff have been instructed to humanely cull it. Animals will be monitored for mobility, piloerection and changes in weight if kept for more than 5 days. Occasionally, it will be necessary to administer anti-malarial drugs to mice for experimental purposes. We will do this mostly by oral administration via drinking water (95% of the time) - this is preferred as it is less invasive.

We will try to use subcutaneous injections if it is a more refined way to infect the mouse as compared to intraperitoneal injections. This is to see which is the most effective and efficient in delivering the parasites into the rodents with the minimum amount of harm and stress to the mouse.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The website for experimental design will be used to give us additional information and help on statistics and experimental design. We will also use NC3R advancements to conduct experiments in more refined way.

The ARRIVE guidelines will help ensure that the data extracted is of high quality, reliable but uses the minimum number of experiments and so reduce animal use.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The official website can keep us informed via email about any advances in the 3Rs and, in conjunction with advice from senior staff in the animal unit, help us implement them if applicable. There are also publications released from time to time reviewing animal models in malaria research which we will also keep up to date with.

79. The use of mouse models to develop better treatments for advanced prostate 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
- 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

Cancer, Prostate, Therapy, Immune response, Microbiome

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 mouse models to better understand how prostate and other types of cancer are supported by the immune system, nutrition, and the microbiome of the patient and to use this knowledge to develop better and kinder treatments for the benefit of prostate cancer 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?

Prostate cancer is the commonest cancer in men living in western countries and despite the high initial success rate, if the cancer is discovered early, the likelihood of a relapse is high with limited treatment options. The advanced stage of prostate cancer spreads throughout the body, often to the bone, the lymph nodes, the liver and other organs. Research leading to a better understanding of tumour biology and therefore to the development of better treatment strategies is an urgent unmet medical need.

For decades, cancer including prostate cancer, has been looked at as a contained disease system, researched in isolated cultured cancer cells. With our research and others, we understand now that there is an interplay between cancer and the rest of the patient's body regarding nutrition, the bacteria helping us to digest food (the 'gut microbiome'), the immune system, and tumour-surrounding, so- called 'stroma' cells.
Research by our lab and others has shown that the immune system can be 'hijacked' by the tumour in a way that helps the tumour to grow, survive and resist treatment. We have also demonstrated how the tumour can be supported by the microbiome, either directly with the synthesis of hormone-like structures and nutritional building blocks needed for growth or indirectly through the manipulation of the immune system. Both determine response to cancer therapies and predict disease progression and survival. We therefore hypothesise that therapies targeting the immune system and/or the microbiome are promising strategies to improve anti-cancer therapies and decrease their toxicity.

However, much remains to be learned about the biology of this interaction and a better understanding of the underlying mechanism, could lead to improvements in patient outcomes.

Our group has developed a strategy to grow small tumour pieces from patient biopsies in mice first, to form larger tumours, called patient-derived xenografts (PDX) which can be further developed in vitro as cell lines growing in petri dishes and transferred again to mice. So far, we have developed over 40 new models covering the most relevant types of treatment-resistant prostate cancer. These have been used in various studies and have greatly contributed to the field already. We would now like to build on this success to study how resistance mechanisms can be prevented or reversed by manipulating nutrition, and the microbiome and thereby impacting the immune system for the benefit of patients suffering from other types of advanced prostate cancer.

What outputs do you think you will see at the end of this project?

To guide our research and to test the impact of treatments on tumour subtypes, our team has developed a method to grow small tissue pieces from patient biopsies in mice. Using these so-called patient-derived xenografts (PDXs), we can predict the success of new treatments. We work closely with drug development research groups as well as with clinicians, in a successful collaboration that has and will continue to help us develop our drug testing efforts and improving on patient care.
We now aim to use this knowledge and our access to donated patient samples to further optimise our tumour models to reflect the advanced prostate cancer dependence on the immune system and microbiome. The use of precise preclinical models to test novel therapeutics is key to drug development, therefore continuous optimisation and expansion of these models is required to reflect the human disease, increasing our likelihood of therapeutic success.

We will use these models to better understand how these cancers become resistant and to develop strategies to reverse resistance and cure patients suffering from the currently incurable disease stage. We will furthermore develop genetically altered mouse strains

that either do not express or overexpress specific cancer-related genes. By studying these models, we can learn to better understand the importance of the role of these genes for prostate cancer growth and survival and this knowledge will help us to develop new drugs and therapeutic strategies to better treat the disease.

These model types will help us to better understand the biology of these tumour types and how to best address them therapeutically. This will increase knowledge in the field as we will publish our results in peer-reviewed journals, present at international meetings and make our findings accessible to other scientists around the world.

Who or what will benefit from these outputs, and how?

Within the term of this licence, the impact will be of great benefit to the field by providing clinically relevant mouse models of prostate cancer to support research that will ultimately have a high impact on patient treatment.

In the mid-term, our research will help to better understand how cancer uses the patient's own resources to grow. At the end of this project, we will have gained and published knowledge on how the surrounding stroma, infiltrating immune cells and the microbiome growth and progression of the tumour and which therapies will be most successful to block this advantage.

This knowledge will inform our decisions in the clinic and will lead in the future and beyond the five- year period to the planning of clinical trials that will ultimately lead to the development of better and kinder treatments for our patients.

How will you look to maximise the outputs of this work?

To ensure the maximum benefits of the outcome of this project, we believe the results and mice developed under this licence need to be shared and discussed with experts and the public. Our group therefore reports regularly at key national and international scientific meetings as well as in high-impact journals.

Where appropriate, we will also discuss our research outcomes with pharmaceutical partners and academic colleagues to ensure positive scientific research findings are developed into clinically useful trials and therapies for patients with prostate cancer without delay.

Our research group also regularly hosts meetings with patients, their families, and patient advocates to update them on our research projects. The results from this project will be reported to them through these open meetings with the community which we serve.

By making our models accessible through our collaborations with other researchers in academia and industry, we will speed up progress and reach our goal to cure prostate and other cancer faster.

Species and numbers of animals expected to be used.

- Mice: 13600

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 genetically and physiologically similar to humans in ways relevant to this study. It is possible to induce human-like prostate cancers in mice by deleting or over-expressing certain genes in their prostate or to grow human tumours in certain mouse strains. These mice, due to a genetic modification, lack a fully functional immune system. In collaboration with clinicians at the hospital, we have already used these strategies to develop more accurate models of tumours in patients that can be treated with different therapeutic regimes. We would now like to use this knowledge to expand our portfolio by remodelling the impact of the microbiome and the immune system, as both have been recently identified as regulators of the growing tumour and are therefore relevant therapeutic targets.

Typically, what will be done to an animal used in your project?

Our team have developed a protocol to generate patient-derived tumour xenografts (PDX). These are implanted under general anaesthesia under the skin or into the prostate of mice, grown, and harvested for further analysis in the lab way before they affect animal well-being beyond the degree needed to achieve the scientific outcome. We will also use mice that have been genetically altered in order to develop prostate cancer predictably.

Some tumours will require a long time to develop, so mice may have to be aged.
In order to study the impact of certain populations of immune cells, in some cases, we will irradiate the mice to deplete them of their existing immune cells which we will transplant with other populations of immune cells by injection.

To mimic the effect of androgen deprivation treatment, a common first-line treatment in patients, some mice will be castrated to reduce the male hormone testosterone in their bloodstream.

Some mice will be treated with substances to test novel treatment regimes. When assessing treatment in mice for the first time, we will perform a small pilot study with a range of drug doses that should not cause harm but will allow us to explore any possible side effects. We will carefully choose drug doses that are effective while minimising side effects.

Blood, faeces, and/or urine samples may be taken to control the effect of treatments. In some cases, this may require housing these mice singularly or the use of metabolic cages.

To investigate the interplay between the gut microbiome and the cancer, some mice will be reared in a germ free environment or will be treated with high-dose antibiotics to remove their gut microbiome which will be replaced with relevant, controlled gut bacteria in order to study their function. In some cases, this may be achieved with an altered diet.

We will monitor tumour growth using non-invasive techniques such as MRI or ultrasound, which will also allow us to observe potential adverse effects earlier and end the study before the mice show debilitating adverse clinical signs. We will use anaesthetic and analgesic regimes and appropriate humane methods of culling.

Treatments are applied orally or by injection. Tumour growth and animal well-being are

carefully monitored before and throughout the treatment.

What are the expected impacts and/or adverse effects for the animals during your project?

All our experiments will ultimately lead to mice that will suffer from cancer, either under the skin or in their prostate. We have much experience in characterising the progression of tumours including using imaging technologies and will ensure the experimental schedule ends before significant spreading of the cancer occurs. Cancers can cause weight loss, impairment of their ability to move, pain and distress. Local tumours can lead to obstruction of the bladder, tumours growing under the skin can hinder the mouse's ability to move or breathe and can impact the function of vital organs.

We will be vigilant for all signs of tumour development. Mice will be checked carefully for signs of tumour growth and distress and will be weighed at least once per week from the earliest point a tumour could develop. Tumours will only be allowed to grow up to a controlled size limit required for the scientific data to be obtained and to ensure they do not develop debilitating adverse effects compromising their activity and behaviour. Tumours and other tissue will be taken to the lab for further studies.
Some of the treatments given will cause side effects such as weight loss. Immune cell transplants can cause unwanted immune reactions and gut transplants can cause diarrhoea or dehydration.

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 the experiments will be mild (55%) or moderate (45%).

What will happen to animals used in 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?

Our aim is to understand the impact of the microbiome and the immune system on the formation and treatment response of prostate cancer. Our team is constantly developing, and optimising methods designed to study individual aspects of this complex interaction. However, as there are limitations in their ability to predict clinical responses we will need to use a biological system (an organism) having an immune system and gut and other bacteria by nature to closer model the biology of cancer in a human patient.

Mice have been proven in numerous studies to be very valuable assets for cancer research and drug development. Their tumours and biology are very similar to human

biology and certain strains can even be used to grow human biopsies (patient-derived xenografts, so-called PDXs). Our PDXs for example have been used to study several drugs that are now in clinical trials helping patients to fight their disease and live longer. We will use immunocompromised mice to transplant human tumour pieces and immune systems and genetically modified mice to study the disease in a more natural environment.

This study focused on the complex interplay between the immune system, the microbiome and different cancer types. We are working closely with experts in all three fields but cannot currently recapitulate such complex interplay of organs and cells within an ex vivo or in vitro system.

Which non-animal alternatives did you consider for use in this project?

We made great progress optimising in vitro assays using isolated cells from our models and culturing them in the lab. These can be used to study the biology and response to various treatments. We also developed methods to grow them in more tissue-like 3D cultures and combine them with cultures of immune cells or supernatant of other cultures. To study the interplay between the tumour and other components of the body, more sophisticated model systems, so-called ‘organs/prostate tumours on a chip’ have been optimised and proven to be very useful tools in the fight against cancer. We have optimised the culture of patient-derived organoids (PDOs) from these PDX lines and have used these for several published in vitro and functional studies. This helped us to develop our biobank of organoid models and to establish a pipeline for downstream analysis and to make the best possible use of the samples obtained in our preclinical mouse work. For this, we have developed expertise in multiplex immunocytochemistry and validated assays for an increasing number of proteins. Our lab continues to work on making these models better and thereby constantly reducing the number of mice needed.

Why were they not suitable?

Despite the effort, these models have many major limitations and key questions are best studied in an organism. Although we have made great progress in developing culture techniques, the in vitro life span of cultures is limited, and we still need the mouse models to propagate the lines and ultimately test a potentially promising treatment strategy in an in vivo model that reflects the tumour structure and dynamics in the patient.

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 based on accurate statistical planning with the help of our statistician, using the experimental design tools provided on the NC3R's website. In order to accurately calculate our numbers, we used data obtained on previous project licences. An example of a typical drug testing study with three doses of a novel drug and a vector control would be, 8 mice per genotype, thus (3 (genotypes) x 8 (mice) x 4 treatment arms = 96 mice will be required for each experiment. We anticipate undertaking approximately

6-8 such experiments per year for every year of this project.

Another 8 mice per PDX line will be needed to grow material for in vitro studies and thereby maintain the line. We will freeze down genotypes that are no longer needed.

During this study, with increased knowledge, we will review and adjust the number of mice needed to obtain the most solid experimental data. Each breeding scheme is calculated based on the information from the strain, the genetic background, and the characteristics of the cohort required (age, genotype, sex). The number of animals required at each stage of the life of the colony (i.e., maintenance, expansion, backing up) will be calculated to minimise the number of animals while safeguarding the quality of the colony over time. The accuracy of the calculation is built on years of knowledge (from previous licences and expertise at the breeding centre). Regular meetings between our teams will ensure that the requests for cohorts are timely and well-defined. Lines of low use will only be bred when required and active mating will not be constantly maintained. Where possible, the line will be cryopreserved and removed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our work is supervised by a trained statistician who is part of our research team and will help us to analyse the experiments, and to ensure that the optimal number of animals per experimental arm is used to obtain a statistically accurate and relevant result. If the impact of a compound on overall well- being or the efficacy in mice is not known, we will perform small pilot experiments with reduced group sizes prior to a larger-scale experiment. We aim to use state-of-the-art experimental planning tools such as the NC3R's Experimental Design Assistant to ensure we achieve statistically robust and relevant results with the lowest number of animals possible.

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 modern non-invasive imaging techniques such as magnet resonance imaging (MRI), luminescence in vivo imaging, ultrasound or X-ray to observe and measure tumour growth and treatment response over time. This will allow us to generate more accurate data and thereby reduce the number of animals needed during the experiment. Accurate planning of the experiment as well as subsequent analysis will ensure that the maximum possible number of parameters will be taken to avoid unnecessary repetition. Genetically altered animals will be obtained from collaboration partners. Efficient colony management and accurate experimental planning will help to reduce the production of unneeded genotypes and to produce accurate numbers of animals to answer the addressed scientific question. We aim for sperm over embryo cryopreservation to preserve the lines and reduce the number of animals needed for maintenance breeding. This may be performed by an external collaborator or contractor.

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 possible, we will utilise in vitro assays to validate the results. However, as these are limited in their accuracy to predict clinical responses, we will use immune-compromised and genetically modified mice. They have been proven in numerous studies to be suitable to investigate human diseases and to be effective predictors of clinical response to treatment. We lay a great emphasis on animal wellbeing and husbandry. To avoid infections, the animals are maintained in areas of high biosecurity. Some mice might be kept in germ-free conditions, an environment without or only with defined micro-organisms. We provide anaesthesia during and pain killers after surgery and ensure the animal stays warm and hydrated during the procedures. Wherever possible, we will try to combine monitoring and treatment to reduce the overall number of conscious interventions. The animals are closely monitored, and tumour burden is kept within defined limits to minimise suffering. If an animal model with a novel genetically introduced mutation shows a phenotype that may cause discomfort or distress, we will aim to develop tissue specific or inducible knockouts that will still allow us to address a given scientific question but reduces suffering of the animal carrying the mutation.

Why can’t you use animals that are less sentient?

Mice are most suitable for preclinical in vivo drug development studies and are widely used for this purpose. We will use optimised husbandry and handling techniques and will make sure that mice are suffering at the lowest level possible by carefully choosing treatment regime and doses. Wherever possible, we will use existing genetically altered mouse strains which have already been shown to be effective in these types of studies. Prostate cancer as most cancers is a disease of older age and needs time and a complex environment to develop. Studies in earlier stages or in anaesthetised mice are therefore not possible.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To avoid infections, the animals are maintained in areas of high biosecurity. The animals are closely monitored, and tumour burden is kept within justified limits to minimise suffering. We will choose non- invasive methods to monitor tumour growth, which will also allow us to observe potential adverse effects earlier and end the study before the mice are showing clinical signs. Wherever possible, we will carry out monitoring and treatments at the same time to reduce stress and the number of separate interventions. Prior to and throughout the treatment, tumour growth and animal well-being are monitored carefully.

We will choose drug doses that are effective but minimise side effects. When assessing a treatment in mice for the first time, we will perform a small pilot study within a range that should not cause harm and explore any possible side effects. We will use anaesthetic and analgesic regimes and appropriate humane methods of euthanasia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

It is important for us to keep up to date with published guidelines the NCRI and ARRIVE guidelines.

Routes and volumes for administration of substances are taken from LASA guidelines (LASA Good practice guidelines for the administration of substances)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We keep updated via the NC3Rs resources hub (https://nc3rs.org.uk/resource-hubs) and use the available tools (e.g. watching recordings of webinars). Our institution ensures we are updated via the NC3Rs e-newsletter and holds regular meetings with the licence holders, Named Persons, and animal technicians to review and implement new 3Rs opportunities.

80. Development of anti-inflammatory therapeutics

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

inflammation, treatment, atherosclerosis, arthritis, diabetes

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 find out if our laboratory developed treatments, inspired by ticks, will work in animal models of inflammation. We will define their effective dose, route, and how frequently we give them.

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 root cause of many diseases. These diseases include heart attack, rheumatoid arthritis, colitis, and type 1 diabetes, among others. The body parts and tissues affected are the heart, joints, gut, and pancreatic islets. In these diseases, the body sends its fighting troops - known as "immune cells" - to destroy the tissues. To allow the tissues to survive, the body's immune cells need to be suppressed with drugs. The cost is that the weakened immune cells are no longer able to fight off infections or cancers. For instance, in type 1 diabetes, despite the best immune suppressing treatment, patients start needing insulin again. Could we simply stop the immune cells from reaching the tissues instead? Body tissues make signals called “chemokines”. These signals act as beacons to call in

the patient’s immune cells. Blocking chemokines could stop the immune cells from reaching their target.
For over 30 years researchers have been trying to make drugs that block chemokines. This effort has not been successful as too many types of chemokines are produced. Blocking just one or two has no effect. Ticks solved the problem millions of years ago through natural selection and evolution. To allow them to feed for weeks on blood without being detected by the host, they inject proteins called “evasins” which neutralize many chemokines. Evasins are effective in animal models of diseases such as heart attack, arthritis, and colitis. Our aim is to develop treatments inspired by tick evasins. These treatments would stop the immune cells from reaching the tissues and destroying them. We recently identified the smallest effective bit of a tick evasin (called a "peptide"). This peptide blocks both major chemokine classes. Our aims are to improve the potency of our peptides and make sure they last longer in the body. Our peptides need to be tested in mice because currently there are no alternative methods to recapitulate the complex immune system responses that we are targeting.

What outputs do you think you will see at the end of this project?

We will have new information regarding the tick-inspired substances that we make in the laboratory, and use them in animal disease models (e.g. artery disease, diabetes, colitis, and arthritis). We will know if the tick-inspired substances will work in these disease models. We will also know how long these substances last in the body, and how often they need to be given. We will publish our data and if appropriate, will patent it. We will then have new potential treatments for treating diseases caused by inflammation.

Who or what will benefit from these outputs, and how?

In the short-term, the outputs will benefit researchers to plan dosing protocols in animal models. These are models of immune diseases affecting blood vessels, heart, joints, gut, and pancreatic islets. We will share this information with other scientists at scientific conferences and by publishing and patenting our data.
In the long term, the potency of the new treatments in disease models will allow studies in patients. This will benefit patients suffering from these diseases. We work with patient groups to understand their needs. We will work with drug companies and clinical researchers to study the potency of these substances in patients.

How will you look to maximise the outputs of this work?

We will maximize the outputs by linking with researchers studying animal models of immune disease. We will disseminate knowledge of both successful and unsuccessful approaches. We will publish in open access journals and present at scientific meetings. We will make available the agents we develop to others for use in their own research. We will raise awareness of our work through patient groups.

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 mice for these studies. The mouse immune system is closer to that of humans unlike other models such as zebrafish. Young mice do not have a fully functional immune system, and so we cannot use them. Our future work on animal models of immune disease will also use adult mice. These models have been used to develop successful treatments, for instance for arthritis and diabetes. The adult mouse is thus a suitable model for this project.

Typically, what will be done to an animal used in your project?

Genetically altered mice will be bred using conventional methods. The mutations these mice carry are not expected to cause any harm that is more than mild in severity.
Some mice will undergo creation of an air pouch by injecting air under the skin on the back. In some animals the fur is shaved so that we can clearly see the area affected. Air injections may happen on two occasions, so that the pouch is maintained. Mice will then have a substance injected into the air pouch that will cause inflammation. Other mice will be given a control substance. Some mice will then be given a substance that has shown anti-inflammatory properties in the laboratory. Within a maximum period of 14 days the mouse will be humanely killed. The fluid from the air pouch will be taken for post- mortem assessment.

In other experiments we will determine an appropriate route and dosing schedule. Some mice will be given treatments by subcutaneous, intravenous, intra-peritoneal, and oral routes. Repeated blood samples will be taken over a maximum period of 28 days before the mouse is humanely killed. The tissues and organs will then be removed for further analysis in the lab. Some mice will have a small device implanted. This allows continuous delivery of drugs rather than undergoing daily injection. This involves minor surgery under general anaesthesia. Mice are given pain relief just like patients after an operation, and are closely monitored until fully recovered.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of animals will have no more than short-lived pain, swelling or local irritation. This may happen for instance as a result of an injection of an inflammatory substance. Small numbers of animals may need placement of a subcutaneous pump to give a treatment. We do this as a minor operation under anaesthetic. It avoids the need for multiple injections. Mice will experience pain until the surgical wound heals. The wound may break down. If this happens, we repair it once under anaesthesia. As a result of pain, there may be loss of appetite, hair standing on end, hunched posture, and mild weight loss. This may happen for up to 48 hours but generally improves within 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)?

We expect that 68% mice will have moderate severity and 32% will have mild severity.

What will happen to animals used in 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 has different cell types. These include neutrophils, basophils, eosinophils, mast cells, monocytes, macrophages, dendritic cells, natural killer cells, B-cells, helper T-cells and cell-killing T-cells. Each cell type has a different function. These cells are called in by the 46 different chemokines to cause inflammation. This makes it a complex system which cannot be reproduced as yet in the lab. The body gets rid of drugs through organs such as kidneys and the liver. We cannot create these organs in the laboratory as yet. We thus need to test drugs in animals to see if they will work, and to see how fast the body gets rid of them.

Which non-animal alternatives did you consider for use in this project?

We use cell-based studies in the laboratory to study if the new drug is likely to block inflammation. We also use organoids to study this. These are collections of different cells that make up an organ, and we make them from stem cells.

Why were they not suitable?

Cell-based and organoid-based studies do not replicate the complexity of the immune system. We cannot use them to find out by what route to give a drug. We cannot use them to find out how long a drug lasts in the body. We need to know this to see how to give a drug to a patient, and how frequently it has to be given.

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 determine this based on previous experiments we have done. We use the minimum number of animals needed so that our findings can be reproduced by others.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We identified the best experiment design from the scientific literature that reduces animal use. We start with the most likely substance, dose and route using information obtained in the laboratory. We do not try all possible substances, routes and doses. We examine the outcome, and then proceed to an alternative if the approach is unsuitable. This tells us if a drug is effective. We then study how frequently an effective drug needs to be given. This reduces the numbers of animals used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Small pilot studies will be done to test efficacy of our drugs. We will develop sensitive assays for estimating the amount of substance in the blood. This reduces the amount of blood taken and allows repeated sampling. It means that in a single experiment we can get much more information and have an accurate representation of how long a drug lasts in the body.

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 mouse air-pouch model of inflammation during this project. We will use this to find out the efficacy, safety, dose, and route we use for a new substance. In our experience, this model causes minimal pain, suffering, distress and no lasting harmful effects. We will use mice to see how long an administered substance remains in the body. The routes used to administer the substances are well- established and not expected to cause more than transient pain, suffering or distress. One of the routes used will be an osmotic minipump that is surgically implanted under the skin, and can deliver a new treatment continuously. Although this step requires a minor surgery, it means that the mouse need not have any further injections. We need to do this step to make sure that new treatments we are developing can actually be given by the osmotic minipump.

Why can’t you use animals that are less sentient?

Less sentient animals such as zebrafish have immune systems that are too different from the human. This means that they will not provide results that are relevant to human disease. Animals at a more immature stage such as very young animals or embryos have immature immune systems. These too will not provide results relevant to adult human disease. These models do not allow us to study how a drug is absorbed or removed by an adult mammal. Drug absorption, and removal takes hours to days. Inflammation also takes days to develop. We cannot study these processes under terminal anaesthesia which lasts only for a short time.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

When we give new substances to animals, they may experience side-effects such as weight loss or distress. To reduce the impact, new substances will be given initially to a small number of animals. Animals receiving new substances will be monitored more frequently to see if side-effects are happening. Loss of appetite and weight loss will be managed by providing soft palatable food such as mash. Some substances are given to cause inflammation and animals may experience pain as a result of inflammation. To

prevent this, small pilot studies will be undertaken to determine the lowest possible dose of inflammatory substance required to generate desired response. Appropriate medication for pain relief will be provided. Following surgery for pump implantation animals may experience pain or get an infection. This will be prevented and managed by appropriate medication to provide pain relief. The risk of infection will be reduced by using sterile techniques.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the PREPARE guidelines and guidance from NC3Rs and Laboratory Animal Science Association to ensure that experiments 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 will sign up to the NC3R’s website to keep abreast of new information and ideas. We are active in developing alternative approaches such as organoid models of inflammation. Through lectures, we disseminate such information to others. We attend regional NC3Rs meetings to learn of best practices and advances in 3Rs.

81. Evaluation of novel devices to be used in surgery

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)

Key words

Surgical devices, Endoscopy, Laparoscopy, Robotics

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?

This project aims to evaluate and further develop novel surgical devices and to identify those which have the potential for use in human surgery. Then to progress these devices, from the bench testing phase, through the necessary pre-clinical regulatory assessments to be registered by, for example, the Medicines and Healthcare products Regulatory Agency (MHRA), the European Medicines Agency (EMA) and/or the US Food and Drug Administration (FDA) for human use.

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?

All devices which are intended for human clinical use need to undergo rigorous safety and efficacy assessment before progressing from pre-clinical to clinical use. Some of these

assessments are to establish which, of several options, is the most appropriate to progress to patient use, and some are to definitively establish safe use of the final chosen instrument(s). All devices under this licence are intended for use in human surgery and are designed to increase positive surgical outcomes, enable new surgical approaches and/or minimise the impact of the surgery itself.

What outputs do you think you will see at the end of this project?

The devices successfully evaluated will be made available to clinicians and surgeons worldwide.
These evaluations should allow rationalisation of potential devices, to defined products, to progress through relevant regulatory bodies, to human clinical use and be used to inform the next generation of a device or new ideas for novel energy delivery.
'Energy delivery' includes the use of microwave-, radio- and ultrasound-frequencies, all of which can be used in patient diagnosis and/or treatment by focusing them in different ways. For example, ultrasound is currently commonly used as a safe, standard method of monitoring a baby's health in the womb however, it is also possible to use ultrasound to treat patients - by using a different form of ultra sound, high intensity focused ultrasound (HIFU), it is possible treat conditions such as uterine fibroids or gall stones.
Several minimally invasive devices (including those for endoscopic, laparoscopic and robotic use) could be registered for human clinical use over the course of this licence.

Most of the work carried out under this licence will probably be for device development therefore publication may be limited by the companies' intellectual property concerns and, as such, it may not be possible to publish it, although there now seems to be an increasing interest in publishing data from such studies.

Who or what will benefit from these outputs, and how?

Benefits should be felt immediately upon the release of devices for clinical use, leading to feedback and the next generation of devices thereby providing long-term benefits to patients who currently need repeat or additional treatments which can extend their dependence and treatment regime.

These evaluations may provide safe additional, or replacement, devices (or combinations) for patients with varying conditions. Many of which could be applicable to several conditions from wound care to complex surgical interventions and thus improve the lives of a wide spectrum of patients.

These benefits may improve surgical outcome thereby improving quality of life for patients together with a reduction in surgical procedure duration, and the related anaesthetic requirements, as well as recovery time and therefore length of hospital stay, which would also decrease NHS costs and free up much-needed bed space. In some cases, these new devices may allow surgical intervention where currently it is not possible, thus increasing the options open to patients with several different medical conditions. For example, previous advances in endoscopic tool and technique development have changed patient treatment from open bowel surgery (requiring significant theatre time and several days hospital stay) to endoscopic surgery (which can be done as a day case, requiring no overnight stay).

How will you look to maximise the outputs of this work?

Where possible, publication in peer-reviewed journals, dissemination at national and international meetings, workshops and seminars. Also, getting new, effective devices/compounds into clinical use as quickly and safely as possible will maximise patient benefits and healthcare savings.

Species and numbers of animals expected to be used

- Pigs: 470

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 device testing, we need an animal of a similar size to humans as the devices to be tested will be the same size as those intended for human clinical use. Juvenile/adult pigs are therefore the animals of choice for these evaluations due to their size and anatomical similarities.

Typically, what will be done to an animal used in your project?

The animals used in these studies will be obtained from a commercial rearer producing animals for the meat industry. Upon arrival in the unit, the animals will be habituated to human contact by hand feeding treats and regular close contact with the animal care staff. The animals will be trained to enter the weighing crate and transport trolley by placing food items in these to encourage them to enter. On the day of the procedure, the animal will be weighed and moved to the induction suite using the transport trolley. Anaesthesia will be overseen by a specialist veterinary anaesthetist and will be induced by the injection of an anaesthetic agent and maintained by gaseous inhalation and mechanical ventilation.
The animal will be moved to the operating theatre and instrumented as required for the monitoring of physiological parameters during anaesthesia. A surgical procedure will be undertaken, by a specialist medical surgeon, in a manner that imitates that which would be performed on humans under the clinical criteria for which the device is intended.
Throughout the procedure, physiological data and data specific to the performance of the device will be captured.

Devices will be evaluated for safety and efficacy.

Initially these studies will be non-recovery and only devices showing good results will be carried through to recovery studies. Some of these results may be obtained with the aid of endoscopic or minimally invasive surgery and/or the use of non-invasive imaging such as X-ray, MRI or ultrasound.

For the recovery studies, this will be done by the anaesthetised animal undergoing surgery in a similar way to that of a human patient. The effects would also be monitored in a similar way, for example using a combination of the following: observation of general health and behaviour, regular blood tests, non- invasive imaging (e.g. x-ray, ultrasound or MRI) or via further endoscopic assessment to visualise the operated area and potentially to also take biopsies. Also, as with human patients, the benefit/necessity of the various monitoring modalities will be weighed against their impact on the patient.

For example, for an endoscopic access to the bowel for simulated polyp removal, under recovery anaesthesia an endoscope would be introduced via the anus and navigated to the appropriate area of the intestine (it may be necessary to flush the intestine at this point to remove any faeces present). The area would be marked, an endoscopic injection used to raise a 'polyp' and the mucosal tissue excised (approximate area: 3-6cm in diameter), this may be repeated several times (to replicate the human clinical situation). The treatment sites would then be checked and the scope removed and the animal allowed to recover from the anaesthesia. Blood samples may be taken pre- and post-operatively as well as at several later time points to assess any change in general blood chemistry. Possible re- scoping time points for an eight week study would be days 0, 3, 7, 14, 28 and at termination this would allow a visual assessment of the treatment site- any necessary blood samples would also be taken at these times. These blood tests and scoping 're-looks' would also serve as a method of monitoring overall animal health and any significant deviations from normal that could cause unnecessary animal suffering would constitute a humane endpoint. As these animals would have no external wounds or cannula there should be little or no need for individual housing. Where possible, blood sampling, scoping and minimally invasive imaging will be carried out at the same (or at least overlapping) time points to minimise the number of instances of anaesthesia the animal has to undergo thereby stressing the animal as little as possible. This should mean that, for a 4 week study, there would be no more than 6 instances of anaesthesia including the initial surgery and at termination.

At the end of the experiment the animal will be humanely killed and the operated site examined and removed for further examination by a pathologist.

What are the expected impacts and/or adverse effects for the animals during your project?

For endoscopic procedures:

From previous experience, we do not expect to see any adverse events. Following complete recovery from anaesthesia the animal should appear normal and show no signs of pain, weight loss, or abnormal behaviour.

For laparoscopic procedures:

Potential adverse effects

From previous experience, and because all devices we need to test will have undergone rigorous bench testing and, where possible testing on dead tissue, we do not expect to see any adverse events. However, post-surgical infection is always a possibility following surgery. This will be specifically monitored and, if it does occur, relevant intervention or treatment under veterinary advice, will be applied. If intervention or treatment is inappropriate or ineffective the animal will be killed using a humane technique.

Avoidance of adverse effects

Good sterile technique and good preparation of the subject before surgery will ensure the absolute minimal chance of infection. Good use of pain relief medication will minimize the possibility of anything more than minor discomfort associated with the surgical procedures. Good monitoring for signs of pain will allow timely intervention under veterinary advice.

For open procedures:

Potential adverse effects
From previous experience, and because all devices we need to test will have undergone rigorous bench testing and, where possible testing on dead tissue, we do not expect to see any adverse events. However, post-surgical infection is always a possibility following surgery, as is wound dehiscence/ suture failure. These will be specifically monitored for and, if it does occur, relevant intervention or treatment under veterinary advice, will be applied. If intervention or treatment is inappropriate or ineffective, the animal will be killed using a humane technique.

Avoidance of adverse effects
Good sterile technique and good preparation of the subject before surgery will ensure the absolute minimal chance of infection. Good use of pain relief medication will minimize the possibility of anything more than minor discomfort associated with the surgical procedures. Good, multi-layer surgical closure will reduce the risk of suture line failure. Good monitoring for signs of pain will allow timely intervention under veterinary advice.

Humane endpoints
If any animal shows unacceptable changes to behaviour or physiology, full consultation with the vet and other local animal welfare staff will be undertaken to determine the best clinical care for the animal. If improvement is unsatisfactory 24-48 hours after any intervention or treatment, the animal will be killed using a humane 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)?

Pigs:
Non-recovery - 32%
Moderate severity - 68%

What will happen to animals used in 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 assessment of safety, effectiveness of surgical devices, requires full biological systems to show that they do not cause any unacceptable reactions in living tissue but that they have the appropriate effect in as 'human-like' an environment as possible. It is not yet possible to accurately and reliably simulate this complete system.

Which non-animal alternatives did you consider for use in this project?

We have considered virtual reality for testing of the devices and we do, where possible, use these systems and other simulations for ergonomic testing and early instrument development. We use tissue from dead animals for all initial tests where possible, but ultimately we need to know that the material or material/instrument combination is safe and effective in a fully functional biological system. As this licence is intended for testing the performance of the entire device, in-vitro and biocompatibility testing will have been conducted before reaching this phase of testing.

Why were they not suitable?

Virtual reality systems are not currently safety or efficacy predictive and as such they are not yet accepted by regulatory bodies. And it is not possible to assess healing, or the ability to stop bleeding, in dead tissue.
It is not, currently, possible to carry out full device testing without using an in-vivo model as there is a need for a complete biological system, especially if looking for histological and/or haematological responses (including testing of coagulation efficacy) and/or longer-term healing. Using pigs for device testing has several well established models which we have used for many years; due to their anatomy and general size/weight, they are currently the most appropriate for testing devices, prior to their use 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?

For all safety and efficacy studies we will reduce numbers as far as possible by utilizing data as predicate where devices/materials are similar and by using as many sites as possible per animal without impacting upon the data obtained or negatively affecting the animal's welfare.

The number of animals is based upon previous device testing studies we have carried out over a number of years where the number of animals used was based on the advice from bio-statisticians. For any significant changes to protocols, further statistical advice may be sought. However, as a rough guide, each full study would normally include 1-5 terminal experiments (usually with low numbers (e.g. n=1-3)), 1-2 short term/pilot recovery experiments (e.g. n= 2-6) and one or more longer term recovery experiments (e.g. n=3-6+, this will depend on the device being tested, the number of sites possible per animal and the number of variables (often mandated/ 'recommended' by regulatory bodies)).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Where possible tissues are retrieved from animals killed following the completion of other studies to reduce the number of animals used for tissue retrieval and instruments will have been progressed through assessments using dead tissue, prior to progressing to full evaluation in a live model. We will continue and expand this philosophy to reduce the numbers of animals needed in the pre-regulatory studies and, where possible, follow the Norecopa PREPARE checklist. Norecopa is Norway's National Consensus Platform for the

advancement of "the 3 Rs" (Replacement, Reduction, Refinement) in connection with 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?

As we retrieve more knowledge from these studies and build an "in house" data bank we will be able to refer to these results thus reducing the 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.

Surgical devices need to be assessed in an appropriately sized animal whose anatomy mirrors that of humans as closely as possible and the pig is typically used for this, especially with respect to the abdomen and gastro-intestinal tract. Safety and efficacy evaluation of the instruments and material/instrument combinations needs to take place in as close a situation to human use as is possible. Some models have been developed by us over several decades of device testing and others can be taken from the literature but there may be some incidences where a model will have to be developed to adequately and specifically answer the study questions. Any models developed to test instruments or materials will take direct note of the potential for discomfort to the animal. As these evaluations are to facilitate translation from pre-clinical to clinical human cases we feel there is little point subjecting an animal to a procedure which would not be well tolerated by a human subject and, as with human subjects, anaesthesia and pain relief will be used to minimise pain and discomfort.

Why can’t you use animals that are less sentient?

For the device testing we need an appropriately sized animal as the devices to be tested will be those intended for human clinical use. The pig is therefore the animal of choice for these evaluations. Also, the majority of the initial testing will be carried out on animals that never recover once anaesthetised at the start of the experiment.

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- and intra- operative care and monitoring will minimise unnecessary suffering. The use of minimally and non-invasive assessment (e.g. MRI or X-ray) as well as scoping, whilst 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 (by allowing internal assessment at multiple time points) and therefore reduce to overall number of animals used. Also, with a degree of animal 'training' and familiarisation, and the correct pre-

medication, the stress/suffering to the animal can be minimised - this applies to medication delivery, acclimatisation to single housing, blood sampling from a cannula and any other events that require interaction with the animal. Time spent 'training' each animal also allows the animal husbandry staff to become more familiar with the personality of each animal and therefore more aware of any changes in behaviour (which is most often the first sign of any systemic change). Also, following open abdominal surgery, the use of a mash/ more liquid diet to aid digestion immediately post-surgery is often recommended. 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 PREPARE and NC3R and ARRIVE checklists coupled with reviews of the current literature and any revisions to the regulatory guidelines along with reference to the LASA guidleines on undertaking aseptic surgery (2017 edition). I have also been referred to standard, established, well regarded reference books, for up to date anaesthesia advice/techniques.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Review of the current literature 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

82. Evaluation of pharmacokinetic properties of novel drug candidates for debilitating diseases with high unmet clinical need

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

Pharmacokinetics, Biofluid sampling, Tissue sampling, New therapies

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?

It is important to undertake this work as there is currently a lack of effective therapies in the disease areas we are researching. The pharmacokinetic (PK) properties of a novel drug (absorption, distribution, metabolism and excretion by the body) can profoundly influence efficacy and will determine its suitability for development as a therapeutic agent for the treatment of debilitating human diseases. The aim is therefore to evaluate the pharmacokinetic profiles of novel agents in mice and/or rats to assess whether these compounds have acceptable properties, allowing optimization of dosing regimens prior to testing in animal models of disease, authorized under separate disease specific Project Licenses.

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 focus on severe diseases with a high unmet clinical need, which are currently nucleotide repeat expansion diseases, polycystic kidney disease and narcolepsy. The work described in this licence application is designed to evaluate the in vivo pharmacokinetic properties of novel candidate drugs (absorption, distribution, metabolism and excretion) in relation to in-house animal models. These pharmacokinetic drug profiles are obtained before testing in animal models of disease, to enable targeting of an efficacious dose range and to ensure the total number of disease model animals is kept as low as possible. Data generated in animals can also be used for human dose prediction before studies are planned in man, thus the data will be used to help progress promising compounds to the clinic. This work will form an integral part of the overall research strategy to identify and develop effective therapies for the treatment of these severe human diseases.

What outputs do you think you will see at the end of this project?

This work is expected to provide:

1) Novel information on multiple pharmacokinetic parameters across our numerous varied projects.
2) Data generated will be used to identify candidates for further development within animal efficacy models of disease, or for human dose prediction.

Who or what will benefit from these outputs, and how?

This project will explore drug exposure parameters in rodents, thereby facilitating the selection of compounds for further development and project progression in our efficacy disease models in the short term. In addition, data from studies may be used for internal stage-gate documents (medium term) and regulatory documents (longer term), which are ultimately required to progress therapeutics to clinical trials in man. Overall, our research projects are expected to identify one development candidate per year in each disease area, from which 2-3 would be expected to enter clinical trials within the 5 year lifespan of the individual Project Licences. This Pharmacokinetic Licence supports all of our preclinical disease area research, providing relevant in vivo project information on compound levels and metabolic characteristics.

How will you look to maximise the outputs of this work?

Findings will be made available to other scientists at presentations and internal meetings to further our understanding in the particular disease area. We will also take as many tissues as possible at the end of studies, which will be made available to other company researchers, thereby allowing the maximum amount of data to be generated from each animal used.

Species and numbers of animals expected to be used

- Mice: 350
- 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.

All the animals used in this project will be rodents. In general, the anatomy and physiology of the mouse and rat is well understood and provides the best compromise in terms of correlating with human biology whilst exhibiting the lowest level of sentience for mammalian species. Generally inhouse Pharmacokinetic studies make use of the same species used for corresponding efficacy studies – which is predominantly mouse. This is because any in-house genetically modified disease phenotypes are in mice and so this is mirrored for PK studies, generally in the corresponding wildtype animals. In very rare instances, genetically altered mice may be used if we suspect their PK characteristics might differ from wildtype animals. An example would be the polycystic kidney disease model mice with a cystic phenotype, which may affect elimination of test compounds through the kidney. The stage/severity of genetically altered animal disease will be critical to reduce potential adverse events from compound dosing, therefore if these mice are considered for PK studies they will be used at a predetermined minimally affected phenotype.

Due to this fact rat models of disease and therefore also rat PK studies are very unlikely to be used in- house.

All studies will be performed in adult animals for consistency across studies and to ensure ADME (absorption/distribution/metabolism/excretion) mechanisms are fully developed within the study species.

Typically, what will be done to an animal used in your project?

Compounds of interest will be administered orally, by injection, or via surgically implanted minipumps (required when a continuous supply of compound is needed).
Repeated biofluid sampling, typically over a 24 hour period, via various routes will then occur, before terminal sampling where tissues may also be taken for analysis. Repeated tail blood sampling in the mouse is the most commonly used PK model in-house, which allows multiple time-point acquisition from the same animal, increasing statistical power and using a very small blood micro-sampling volume (10µl), which is less impactful to the individual. In the very rare case urine collection may be requested, the least impactful method will generally be employed, eg. place mouse on Lab Sand and collect urine by pipette.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals are expected to experience momentary pain and stress during administration of therapeutic agents. Animals will experience some discomfort after surgery, but appropriate pre-operative analgesia and post-operative care should minimize pain and aid recovery. Serious adverse effects are not expected, but if they occur, are likely to involve body weight loss and deterioration in clinical signs. Any animals exhibiting such signs, which cannot be ameliorated, will be killed promptly and 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)?

Mice - 85% mild and 15% moderate.
Rats - 90% mild and 10% moderate.

What will happen to animals used in 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?

Computer modelling and in vitro screening provide useful information on properties of candidate compounds. These studies, however, are not sufficiently accurate at predicting the behaviour of compounds in vivo, as pharmacokinetic properties are the result of complex interactions within the body that cannot be replicated in vitro. Ultimately the relationship between compound efficacy and the levels present in different tissues is driven by compound absorption, distribution, metabolism and elimination, and cannot be accurately modelled in vitro. Therefore animal testing is essential to allow discovery of these compound properties.

Which non-animal alternatives did you consider for use in this project?

Compounds of interest will be screened initially in a series of cell assays specifically designed for each proposed target. This will determine the compound has high potency against, and selectivity for the molecular target. An early assessment of pharmacokinetic parameters will be made in parallel to the determination of cellular activity. Assays available for in vitro pharmacokinetic evaluation include metabolic stability in multi-species liver microsomes and/or hepatocytes, ability to inhibit cytochrome P450 enzymes and Caco-2 cell permeability.

Why were they not suitable?

The in vitro assays provide useful screening information to triage compounds for use in vivo, but cannot provide the full wealth of information required to allow a compound to be tested in an in vivo efficacy model, which may then ultimately provide human dose prediction information for clinical trials. Due to the increasing use of inhouse in vitro pharmacokinetic screening, coupled with a high proportion of initial in vivo PK studies being conducted out of house and based on previous animal numbers used on this license in the past, the low numbers quoted below are anticipated.

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 requirement for PK studies can vary depending on different project needs at the time, so numbers based on previous work and experience in this area have been used. Optimisations over previous years have led to a typical study design longitudinally sampling 3 mice at each dose level, resulting in 9 mice being typically used to test 3 doses of a project compound of interest. Longitudinal tail micro- sampling is implemented to minimally impact animal welfare, as well as keeping the total number of animals required to acquire the necessary data to an absolute minimum. This maximization of PK data capture from a single animal, coupled with animal usage based on the work required to support testing one potential therapeutic compound per year in our disease models, over the 5 year licence period, keeps total PK animal usage low.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have access to an internal Preclinical Statistics Centre of Excellence (CoE), which is a team that assists with experimental design, data analysis and reporting of results. Increased bio-analytical sensitivity means that smaller samples can be used to give reproducible meaningful data. This coupled with repeated sampling in the same animal (e.g. tail sampling in mice) will reduce the numbers required for each study. Extensive in vitro testing is also undertaken, which filters out compounds with the most favourable characteristics prior to in vivo testing.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The vast majority of PK studies are performed in wild-type animals, which are ordered in specifically or used from wildtype littermates when required. Colleagues with expertise in drug metabolism work closely with us and we share tissues with them to optimize PK readouts and 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.

Mice (and potentially very rarely rats) are used as they are the lowest suitable species where data can be extrapolated to humans. Repeated tail sampling in the mouse is the most commonly used PK model in-house, which allows multiple time-point acquisition from the same animal, increasing statistical power and using a very small blood micro-sampling volume (10µl), which is less impactful to the individual. During studies animals are group housed to mitigate isolation stress. Rats may be very rarely utilized, but again will be group housed and low volume serial sampling implemented. PK study duration is short, with typical completion after 26 hours. Guidelines limiting the volume and frequency of substance administration and sampling will be strictly adhered to. In the case of urine collection the least impactful method will generally be employed, eg. place mouse on Lab Sand and collect urine by pipette. In very rare instances, genetically altered diseased mice may be used if we suspect their PK characteristics might differ from wildtype animals. An example would be the ADPKD model mice with a cystic phenotype, which may affect elimination of test compounds through the kidney. Animals will be closely monitored over the course of the studies. Humane endpoints have been discussed with the named vet.

Why can’t you use animals that are less sentient?

In general, the anatomy and physiology of the mouse and rat is well understood and provides the best compromise in terms of correlating with human biology, whilst exhibiting the lowest level of sentience. Adult animals are required to ensure ADME (absorption/distribution/metabolism/excretion) processes are fully matured and functional. For dose routes such as oral, a conscious animal is a necessity to avoid aspiration of the dose.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Regular evaluations of procedures and their associated welfare implications will be conducted throughout the project. Findings will be discussed in the context of improvements that can be made without impacting on scientific outcomes at AWERB meetings and bi-annual licence inspections. We will also seek advice from the NACWO and NVS, as well as other scientists working in the in vivo facility. The majority of PK studies are over a short period (24hrs) using an established protocol known to minimally impact the welfare of subject animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will refer to published guidelines issued by NC3Rs as well as LASA (Laboratory Animal Science Association) and ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines, to ensure our studies are performed in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed by consulting the NC3Rs website (https://nc3rs.org.uk/), attending relevant talks and conferences e.g., the NC3Rs Pint of Science events and consulting with colleagues. 3Rs information is also distributed by the Named Information Officer and the Named Training and Competency Officer. We will liaise frequently with the Named Veterinary Surgeon (NVS) and Named Animal Care and Welfare Officer (NACWO) to get advice on how to implement any advances.

83. Pathology and treatment of lysosomal and related disorders

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Lysosomal disease, Inflammation, Central nervous system

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 purpose of this project is to investigate a set of rare diseases that affect a part of the cell known as the lysosome, a fundamental compartment in many cells. We will study the impact of the immune system on the central nervous system (CNS) during disease course to help develop new targets for future therapies in murine 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?

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 can result in widespread inflammation, modulation of diverse signalling pathways, organ damage 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 primarily affect the brain making treatment development very challenging. 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 B since enzymes are unable to enter the brain. Bone marrow transplant is curative for a small subset of lysosomal diseases, but not all. 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?

Outputs from the project:
1) we will acquire a deeper understanding of the pathological mechanisms (with particular focus on the immune response) to LSDs in mouse models relevant to human disease.
2) we will understand if the immune response can be modified to improve neurological decline and neuropathology in LSDs.
3) we will publish the results in open access, high-quality scientific journals and present the work at relevant conferences.
4) we will share our results with collaborating scientists and ultimately make our data publicly available.

Who or what will benefit from these outputs, and how?

There are currently no adequate treatments for these conditions, as such the main benefits of this project will be to broaden the understanding of disease mechanism (with a focus on immunological pathways), and downstream of this the testing of novel treatments that could make a significant impact in the lives of these children. The work will assess complimentary treatment strategies which are targeted to the brain and help to bring them to clinical trial more rapidly.

These data will be of paramount importance for the development of a novel complimentary therapy for lysosomal diseases in the next 5-10 years and in the short-term will inform neurologists and immunologists both nationally and globally in the interim with our research findings on the role of CNS immunity in LSDs. 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 alternative therapies 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 externally to generate and share knowledge of these diseases and models. We will look to publish all findings in open access journals, even when unsuccessful. We will disseminate knowledge through internal seminars, as well as national/international conference presentations and publications.

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 the least sentient species in which one can still model human disease progression of lysosomal diseases. Mice used will typically be between two 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. Though it is clear that differences exist between mice and human brains in terms of structure, function and geometry, there is still substantial similarity in the physiology, including the immune and inflammatory response.

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 diseases and immune models may start to show behavioural changes, reductions in joint mobility and evidence of inflammation, depending on the disease and most have a shortened lifespan. We closely monitor harmful effects on animals and introduce mitigating measures where appropriate or humanely killed animals where required. Typical protocols include:

1) Phenotyping (measuring behaviour, blood and tissue samples) 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 (10-60 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. Samples of bodily fluids (blood, urine) may be taken to analyse disease progression, effects of treatments and/or the health of the animal on up to 10 occasions. Animals may be killed under terminal anaesthesia to allow their tissues to be collected.

2) Delivery of multiple therapies: In addition to phenotyping tests above: mice may receive up to four therapeutic substances by multiple routes (oral gavage, intravenous, intraperitoneal, subcutaneous, intracranial, intracisternal) via up to 12 injections, where no more than 2 injections can be given in 24 hours. Therapies may also be given via a non-invasive route (through diet or drinking water). E.g: An animal receives an anti-inflammatory substance in drinking water (non-invasive), then up to 4 peripheral injections of a disease modifier, and receive 6 injections into the brain of a therapy under anaesthesia. They may then receive further injections of therapeutic substances. Typically, phenotyping will happen after these steps, but may happen prior to drug administration.

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 harvested for further analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Expected adverse effects include weight loss (weight loss will not exceed more than 15% of previous week's weight), 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 (100% incidence). Injections directly brain or the cisterna magna, a fluid filled region at the back of the brain, are performed under anaesthesia, as a result animals have few associated adverse effects. Local bleeding is possible after surgical incision of the skin. Infrequently seizures or brain bleeds can happen in the initial hour after surgery. There is potential for 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)?

Expected severities will be mild to moderate. In breeding protocols 50% of mice will experience subthreshold harms, 50% mild. In experimental protocols 40% will be mild and 60% moderate.

What will happen to animals used in 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 conditions we work on are complex and are influenced by variety of factors. Many of these conditions are neurological in nature but also have non-neurological components often including heart problems and bone disease. As such, non-animal alternatives, such as in vitro assays, may not be appropriate as they can only provide limited results. The mouse models of these conditions mirror the human disease closely, giving us not only physiological data but also behavioural outcomes, 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 treat affected organs, in particular the central nervous system, so computer based modelling and in vitro assays cannot predict outcomes. There are no appropriate alternative methods to avoid the use of animals to assess the impact of drug treatment on CNS immunity, due to the complexity of CNS cell-cell interactions and more generally immune responses.

Our therapies and understanding of pathology will be assessed with a variety of outcome measures such as behaviour and tissue sampling which can only be achieved with animal models.

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 candidates to viable numbers for in vivo testing by aiming them at specific characteristics - specific immune cell targeting. We have considered the use of organoids and brain stem cells from patient derived induced pluripotent stem cells (iPSCs) to select aid in selection of drug candidates, and while immunological and other biochemical/neuro- physiological responses can be measured, we would not be able to measure the impact on behaviour and other phenotypic outcomes. There are no suitable in vitro assays or alternatives that are therefore relevant and combine a model which allows us to assess delivery of products, immune system-CNS responses and behavioural outcomes.

Why were they not suitable?

The CNS 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 typically yielding the proportion of one unaffected, two mixed and one affected mouse. All unaffected and affected mice will be transferred to other protocols. Typically, we will maintain up to five rodent lines at any time, the remainder being kept as frozen stock. From historical experiments, we can estimate that we will typically need 10-12 mice in each group (typically 3-4 groups) to see differences between treated and untreated mice (by analysing behavioural outcomes and biochemical markers/immune composition in bodily fluids/tissue). We also have an accurate idea of how many experiments we would like to complete over the course of the five years that this licence will run. We expect to evaluate the role of multiple pathways and therapeutic substances (targeting 5-6 immune pathways/cell types).

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 plan to share controls between experiments where possible by running multiple 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. The number of animals born from genetically altered animals will be reduced by following good colony management strategies described on the nc3rs website, such as holding stock but not breeding, and intermittent breeding when necessary.

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. Where possible we will utilise sampling techniques throughout the lifespan of the animal, reducing the need to sacrifice animals. We have a number of collaborators, therefore, maximum use is made of animal tissues via tissue sharing across a number of different projects.
Pilot studies will be undertaken when starting new experiments to inform experimental design, i.e. power calculations, again optimising 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.

To achieve the objectives set out in this project licence we will need to breed different mouse models. These will include transgenic mice which allow manipulation of immune cell populations (cytokine/receptor deficient animals) as well as, but not limited to the mouse models of mucopolysaccharidosis (MPS) type III and Gaucher disease. 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 similarities between the metabolites accumulated, display different behavioural and biochemical outcomes. Comparisons of these models will help us to understand which components (with focus on the CNS) are important in each diseases' progression. Immune cell/cytokine deficient models will in some experiments be bred with disease models to assess the contribution of various immunological pathways to disease progression. These disease models will also be used to test the therapeutic effects of candidate drugs, particularly aimed at modulating the immune response and highlighting potential mechanisms and therapeutic targets.

Why can't you use animals that are less sentient?

Rodents provide some of the best available models of human disease progression as characterised previously. Other models, such as zebrafish, are inappropriate as abnormal neuro-cognitive phenotype 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. Many regulators do not accept 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.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Surgical procedures are well tolerated (less than 1% mortality) in lysosomal disorder models and this is in part due to the efficiency with which we can complete the procedure given our experience with the model (typically under 30 minutes).
All procedures are undertaken using good aseptic technique to minimise the risk of infection. Post- operative care will involve increased 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, which includes acclimatisation periods to researchers and test arenas.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All relevant NC3R's guidance and updates will be engaged with, including sign posting to published studies, e.g. FRY, D. 2014. Chapter 8 - Experimental Design: Reduction and Refinement in Studies Using Animals. In: TURNER, K. B. V. (ed.) Laboratory Animal Welfare. Boston: Academic Press and the ARRIVE guidelines for reporting animal research (Kilkennyet al., 2010) - Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving bioscience research reporting: the PREPARE Guidelines for reporting animal research. PLoS Biol 8:e1000412

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 also attend periodic seminars and other educational events hosted within our animal facility. All our experiments will be conducted following PREPARE guidelines, ARRIVE guidelines and OECD protocols to further this knowledge as well as reading publications and outputs of colleagues.

84. Non-invasive monitoring of bovine metabolism

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

Key words

dairy cattle, metabolic disease, microneedle, monitoring, non-invasive

Animal types	Life stages
Cattle	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 whether a non-invasive microneedle patch placed on the skin of cattle can be used to monitor for biomarkers of metabolic disease in cattle.

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?

Dairy cattle are prone to diseases after calving. This is largely due to the abrupt increase in dietary energy requirements for milk production. Monitoring for these diseases typically requires taking a blood sample, which requires technical skill and is an invasive procedure. We propose to determine whether an inexpensive microneedle skin patch could be used in place of invasive blood sampling for the monitoring of metabolic diseases of dairy cattle.

What outputs do you think you will see at the end of this project?

Outputs will include:

- New information on the feasibility of microneedle technology for monitoring of metabolic diseases in dairy cattle.

- Publication of our findings in a peer-reviewed scientific publication

Who or what will benefit from these outputs, and how?

In the short-term, we will use the findings of this study to determine whether to pursue further research funding to undertake more scientific investigations to validate our initial findings. We may also expand the range of biomarkers of metabolic disease assessed.
In the long-term, our findings may improve the health and welfare of dairy cattle by developing a novel and non-invasive technology for the monitoring of metabolic diseases of dairy cattle.

How will you look to maximise the outputs of this work?

This project is a collaboration between two disparate areas of expertise: biomedical engineering and a veterinary researcher. As a result, our findings will be disseminated among a multi-disciplinary audience. We plan to publish our results regardless of whether our approach was successful in determining the feasibility of this novel and non-invasive technology.

Species and numbers of animals expected to be used

- Cattle: 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.

Dairy cattle are susceptible to multiple metabolic diseases post-calving as they transition from being a non-lactating "dry" cow to a lactating "milking" cow potentially producing over 40 L of milk per day within weeks of calving (giving birth).
For this reason, we will be focusing on dairy cattle that have recently calved.

Typically, what will be done to an animal used in your project?

This project involves jugular vein catheterisation for the purpose of collecting serial blood samples and placement of a microneedle patch on the skin.
Cattle used in this project will be temporarily restrained to allow the jugular vein to be catheterised. A small (roughly 5 x 5 cm) patch of skin will be clipped and a microneedle patch placed inside.

What are the expected impacts and/or adverse effects for the animals during your project?

Cattle will experience momentary pain associated with catheter insertion. Catheterisation may also cause mild pain associated with localised bruising. This pain will last no more than several hours. There is a risk of causing localised bleeding around the vein during the process of venipuncture. If this occurs, swelling will be minimised by providing manual compression of the area. The swelling will resolve within several days.
Microneedle patches are non-invasive as they only penetrate the outer (intra-dermal) layer of the skin. They cause a skin imprint lasting up to approximately 30 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 greatest severity in this proposal is mild and is associated with jugular vein catheterisation.

What will happen to animals used in this project?

- Kept alive at the establishment for non-regulated purposes or possible reuse
- 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?

We need to use animals because the ultimate goal of our research is to develop non-invasive monitoring technology. We do not and can not know, however, whether this is feasible without the development and testing of the technology using animals. Research has already been undertaken using this technology on humans: glucose monitoring for example. We now need to determine if this technology can also be used to benefit animals.

A search of three databases (PubMed, Web of Knowledge, Google Scholar) indicates that our proposed study has not been undertaken before.

Which non-animal alternatives did you consider for use in this project?

- Computer modelling can be useful where sufficient information is known to predict what is likely to happen, but it does not explore the underlying causal mechanisms.
- Lower-order invertebrates which also have complex physiological systems

Why were they not suitable?

- Computer modelling: Insufficient information exists for this to be possible.
- Lower-order invertebrates: Their physiological processes are too far-removed from our target species of interest (livestock species) to be of merit.

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 preliminary study to determine the feasibility of the technology in live animals. We have conducted studies on recently deceased cadavers and this has demonstrated that skin measurements are feasible. We now need to test the technology in live animals with parallel blood samples.

A study by Vieira et al. (2010) used 10 non-lactating dairy cattle to evaluate serum concentrations of nonesterified fatty acids, glucose, insulin, and progesterone in response to a glucose infusion. They were able to determine a statistical difference in blood biomarkers between treatment groups (saline vs glucose infusion). We have based our numbers on this and anticipate that we may need more animals to detect a difference in skin levels. We also wish to conduct the studies on both beef and dairy cattle.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A number of steps were considered to reduce the number of animals being using in the project:

- We evaluated the literature to determine the most appropriate sample size.
- We sought opinion from collaborators with expertise in microneedle technology and specialist statistical advice during the study design process to determine the number of animals needed to meet the outcomes of the project.
- Online tools, such as the EDA NC3R's, were appraised for sample size advice for projects without a formal hypothesis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All data generated from the pilot study will be shared in open-access online databases. There will be multiple peer-reviewed publications and presentations at scientific and farmer conferences. New knowledge will also be shared via social media and industry news outlets (such as British Cattle Veterinary Association, Farmers Guardian and Farmers Weekly).

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.

Cattle will be studied using the most minimally invasive procedures needed to meet the project aims for multiple reasons:

- Metabolic diseases commonly occur in cattle, particularly post-calving. The new knowledge resulting from our study will may help new technologies that will improve bovine health and welfare.
- We will be using commercial cattle that are used to being handled. The cattle will remain on their source farm and return to the herd after participation in the study This means that they will find it lessstressful than an equivalent-sized large animal model.
- Cattle are sufficiently large to obtain the required measurements. The techniques may be more challenging to perform in smaller species such as companion animals.
- Methods will include jugular vein catheterisation.

Why can't you use animals that are less sentient?

Less sentient species would not allow us to meet the aims of this study for multiple reasons:

- They are not the end-beneficiary of this research. The technology is intended for use on cattle to monitor for metabolic disease.
- Less sentient species are too small for the technology to work.
- Less sentient species have different anatomy and physiology so the results would not have inferences for our species of interest: cattle.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are already using the least minimally invasive procedures possible so we believe that it will be difficult to minimise these further. If however, we learn of methodology to refine our procedures further, this will be implemented if it does not invalidate the data already obtained.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are various checklists available for reporting studies, such as the ARRIVE guidelines, and these have been used in the planning of our studies. We have used, and will continue to use throughout the study, the PREPARE guidelines, which provide a little more emphasis on items such as harm-benefit, health monitoring and fate of the animals. We will also monitor NC3Rs for updates.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will strive to improve our understanding of the 3Rs by using the available resources, such as the NC3Rs website and News Bulletins, and attending regional events where possible. New information that could improve the welfare of the calves in our study will be used to make changes to our studies where possible.

85. Identifying the biological mechanisms by which the brain-gut-microbiome axis mediates adaptation to psychosocial stress across the lifespan.

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

mental illnesses, neurogenerative diseases, brain-gut-microbiome axis, dysbiosis, neurotransmitters

Animal types	Life stages
Mice	neonate, 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?

Our prior work, and others, indicates that brain disorders for which psychosocial stress is a risk factor, such as mental illnesses, addiction and dementias, also include significant changes in gastrointestinal (GI) and gut microbiome function, yet the precise mechanisms that integrate the functioning of these diverse systems, in health and disease, are not fully understood. Therefore, the overall aims of the project are to determine the biological mechanisms by which stress manifests physically within the body, resulting in changes to genes, proteins and cells of the brain, GI tract and its resident bacteria, which collectively form the brain-gut-microbiome (BGM) axis.
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 know that the stress response is a conserved series of complex biological processes throughout many species that represents a fundamental mechanism for detecting changes within our internal and external environments. As such, it is essential for the survival of

many species. In most cases, the stress response results in biological changes that allow for adaptive responses to such changes; this is called homeostasis. However, depending on the degree of stress (acute or chronic), and at what period in your life you experience it (young; old), stress can induce maladaptive responses which confer a risk for developing a range of debilitating medical conditions. Whilst the brain has long been considered the primary modulator of emotional stress, emerging evidence indicates that other organs within our body are instrumental in influencing such mental states, none more important than the GI system. This is because of the very strong, direct, anatomical and physiological connections between the brain and gut. Crucially, the role of the bacteria resident within the intestine, termed the gut microbiome, have emerged as key players in not only influencing gut function, but extraordinarily, also the functioning of the brain. These systems collectively compose the brain-gut-microbiome (BGM) axis. This joint consideration of the brain, gut and bacteria in terms of stress biology is helping us to better understand why so many stress-related brain disorders, such as anxiety and depression, commonly present with comorbid GI components as well, such as dysmotility, irritable bowel syndrome and inflammatory bowel disorders. Importantly, further evidence indicates that dysregulation of the BGM axis has a negative impact on a variety of other organ systems. This can lead to other negative health outcomes, including cardiovascular diseases such as high blood pressure, metabolic disorders such as type two diabetes, and immune disorders such as arthritis. Therefore, to develop therapies that deal effectively with the causes of such conditions, rather than merely treat their symptoms, it is essential to understand the full biological role of the stress response in altering the underlying processes which lead to the pathology.

Thus, a primary objective for the scientific field is to understand how the nervous systems of individual organs act in concert to bring about a coordinated response to emotional stress for the whole body. To achieve this, it is imperative to study the neural processes of intact, different organ systems, in animals exposed to stress. This will help us to understand the biological basis of such medical conditions, at the whole patient level, rather than the current practice of attending to individual symptoms.

What outputs do you think you will see at the end of this project?

The primary outputs will be:

- New scientific information that contributes to our greater understanding of stress biology and how this contributes to various diseases, will be disseminated by publishing in peer-reviewed scientific journals and by presentations at scientific conferences and Lay public events.

- Scientific justification for future drug development studies and treatment modalities such as bespoke psychological counselling programmes focused on treating stress-induced medical conditions.

- Patents for new drug targets.

Who or what will benefit from these outputs, and how?

SHORT TERM:

- Basic biomedical scientists and clinicians in various medical disciplines (neurology; gastroenterology; metabolic diseases): advancement of basic neurobiology knowledge and how this underpins the pathology and symptoms of medical conditions.

- Drug development scientists: identification of novel drug targets for potential translational exploitation.

LONG TERM:

- Healthcare policy makers (e.g. NICE): translation of the basic scientific knowledge into and the new pharmacological therapies it gives rise to, will be used in the rational decision-making for optimal healthcare policies.

- Patients suffering from various stress-induced disorders will benefit from personalised drug therapies which are capable of addressing the fundamental causes of their condition, thereby modifying the core disease, rather than simply treating the symptoms.

How will you look to maximise the outputs of this work?

All this work is conducted with external collaborators as well as Lay research monitors associated with my Funders. Therefore, I will use these extensive networks to disseminate the knowledge. I will strive to publish the data, both positive and negative, in the most widely read scientific journals.

Species and numbers of animals expected to be used

- Mice: 6500

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 overall aims of the project are to study how emotional stress alters the functioning of various mammalian organ systems. Mice will be used as the broad anatomy and physiology of this species is well understood and provides the best compromise in terms of correlating with human biology whilst exhibiting the lowest level of sentience. Furthermore, the project aims to explore the biological consequences of psychological, rather than physical stress. It's therefore necessary to use an animal model with the cognitive ability to sense psychosocial stress that is imparted by a con-specifics, rather than the investigator. Mice are ideal because of their levels of cognition and how these are instrumental in establishing their social bonds with one another.
Since the aim is to understand the effects of stress at different periods of life, it is imperative to use animals at different ages. Therefore, we will use both neonatal and adult mice.

Typically, what will be done to an animal used in your project?

Broadly, animals will be exposed to stress, primarily by altering their interactions with other mice.

In some instances, mice will have their genes altered, by either removing a gene for a neurotransmitter receptor, or adding genes which contribute to specific disease processes such as dementias. These genetically modified animals will then be exposed to stress, in

order to determine how such genetic changes alter the stress response or disease phenotype.

The project can broadly be divided into two phases, namely the induction of stress in mice, either normal or genetically altered, and then the investigation of the biological consequences of the stress responses in these mice.

INDUCTION OF STRESS:

For early life stress, the behaviour of the mother will be altered which results in a fragmented mother- pup relationship. This impairs the quality of the care she imparts to her offspring, resulting in an enhanced stress response in adulthood.
For adult stress, test mice will be exposed to a more aggressive strain of mouse which results in social subordination and a stress response.

ASSESSMENT OF STRESS RESPONSE:

To determine what consequences stress has on a range of organ systems, a range of techniques will be used, for example:

- Changes in physiology: under anaesthesia, brain activity and how it changes in animals exposed to stress will be determined using standard recording techniques.

- Changes in genes, proteins and cells: animals will be killed humanely and the stress-induced changes in a variety of biological processes, within different organs, will be determined.

- Behavioural assays: stress induced changes in native mouse behaviour will be determined using a range of behavioural tests to assess any anxiogenic/depressive-like behaviours.

What are the expected impacts and/or adverse effects for the animals during your project?

All the adverse effects will be as a result of their exposure to stress or genetic modifications. This can result in changes in:

- Behaviour: animals may exhibit an increased fear response, for example agitation to normal handling.
- Metabolism: animals may have altered body weights due the effects of stress on various hormones or their motivation to feed.
- Impairment in cognition: for the genetically modified animals that model dementias, an age-related decline in cognition is expected.

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% Moderate, due to exposure to stress
60% Non-recovery, since they are used solely molecular/histological analyses following terminal anaesthesia.

What will happen to animals used in 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 the project are to elucidate how life experience, in the form of stress, alters the coordinated functioning of various body systems, such as the brain and peripheral organs, resulting in changes in the behaviour of the animal. The research questions necessitate the use of native animal tissue since the anatomy, physiology and neurochemistry of different neuronal circuits in specific parts of the brain and native animal behaviour can only be elucidated by studying the intact animal itself.
Furthermore, to investigate the changes that occur in brain function due to conditions such as stress or depression, it is necessary to expose animals to such conditions and then determine the consequent effects on the brain itself.

Which non-animal alternatives did you consider for use in this project?

- Cell-culture: It is possible to assess the impact of certain brain proteins on cellular activity by expressing the proteins in cultured cells.

- Computation neuroscience: as scientific knowledge advances, there is a growing availability of computational models of brain function.

Why were they not suitable?

- Understanding the intrinsic mammalian biology of complex, individual organ systems, such as the nervous and GI systems, individually, and in an integrated manner, can only be studies in intact animals, rather than artificial models. This is because brain and GI function, and the associated diseases, arises from a multitude of individual cell types (millions), many of which are yet to be defined. It is therefore simply not feasible to faithfully replicate the molecular, morphological and functional complexity of these different types of brain and GI cells, in basic in vitro cell system.

- Modelling: in order to get robust models, it is essential to feed robust primary data into the various algorithms. The lack of available primary data regarding the effect of stress on the parameters under investigation in this project precludes the use of such technology at this stage.

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 are largely based on the numbers used during similar experiments for my last three Project licences. However, the following refinements have been added:

- When pilot data provided an effect size, I used this to calculate the optimal sample size for individual experiments.

- However, some of these experiments are novel and at this stage, it is unclear what the outcomes will be.

In such incidences, we have been guided by the minimal number animals normally required for associated techniques.
Finally, we have considered the additional animals required when experiments fail as a normal consequence of scientific research as well as the breeding of genetically altered animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

- I have made use of all local support including our statistician and other resources such as the ARRIVE or PREPARE guidelines.

- I also make extensive use of publicly available Big Data resources, in which previously published, primary research data (transcriptomics; proteomics; metabolomics; brain electrophysiology arrays) are available for analyses relating to 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?

- Together with my collaborators, we will use our experience, demonstrated in numerous publications, to use the minimum number of animals which are required to provide scientifically valid data. Since my collaborators also work on body systems other than the brain, we will utilise multiple organ systems from individual animals thus resulting in a reduction of the total number of animals used.

- Together with collaborators, we aim to bank research material (e.g. frozen mRNA; tissue) that can be used by other investigators throughout our networks.

- Furthermore, especially for genetically altered mice, we employ intermittent breeding to ensure that animals are bred to demand, thereby minimising wastage from colonies that are not being immediately used. We also maintain repositories of any surplus tissue for later use by us and 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.

I will be using mice and models of psychosocial stress in:

- : social defeat model of chronic stress

There are a range of models of chronic stress models, which invariably require the animal to be exposed to an aversive experience. The model I will employ is the most refined. It involves exposing the animal to a conflict situation with another mouse, resulting in subordination, or Social Defeat. This initial interaction can last anything from 30 seconds to 1 minute, after which the animals are separated and the stressor terminated. This ~ 1 minute stressor is then repeated daily, for up to 10 days. As such, the model is highly refined to allow for the induction of psychological stress with the minimum amount exposure to stress.

- Early life stress (ELS): limited nesting and bedding mode.

  Again, a range of ELS models require the exposure of the mouse pups to extended periods of separation from their mother, during the very vulnerable first two weeks of life. The model I will employ does not alter the amount of time the mother spends with the pups. Instead, the model replicates the importance of the quality of maternal care, rather than the amount of maternal care. As such, the model directly addresses the most important factor in terms of ELS, without inducing any additional harm to the animals.

Why can’t you use animals that are less sentient?

The aim is to understand how the perception of stress, at the mental level, alters the functioning of various body organs. As such, for most of the project, it is important that a certain level of sentience is required to mentally perceive the adverse experience, so that the emotional limb of the stress response, rather than a basic reflex reaction is elicited. This therefore precludes the use of other animals such as fish or amphibians.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

At all stages of the project, experiences will be monitored to determine whether aversive procedures can be minimised without adversely affecting scientific outcomes.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will seek out the guidance of my NACWO and fellow scientists in the field in order to ensure measures of refinement are always optimal.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will maintain an up to date knowledge of advances in the field, using the conventional routes of scientific articles and conferences, alongside local expertise in the form our NACWO and Named Veterinary Surgeon.

86. Investigating immune responses in mice

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

antibodies, affinity, immunisation, therapeutic, vaccination

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?

During this project we will immunise mice to increase our understanding of mechanisms of antibody generation and maturation in response to antigens.

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 application of monoclonal antibodies in treating both microbial diseases such as pneumonia, bacteria in the blood stream, and post-operative infections, as well as non-microbial conditions like asthma, cancer, and neurological disorders, is on a steady rise. In parallel, the demand for vaccinations, a significant public health measure underscored by the recent triumph of Sars-CoV-2 vaccines, is escalating, aiming for improved results for a broader patient population.

However, the production of these cutting-edge medicines still presents many of challenges for the scientific community. For many new disease targets, their type and nature can

make generating the right antibodies difficult. Additionally, for vaccines, the variability of pathogens adds another layer of complexity, as it requires the development of adaptable therapeutic strategies to generate antibodies that bind to all variants.
The challenge lies not only in understanding the basic disease and immune response mechanisms but also in translating this knowledge into practical applications that can lead to the creation of more effective and targeted medicines. This is, in part, due to the numerous unresolved fundamental questions surrounding the generation of antibodies and the potential manipulation of this process for better outcomes.

Understanding how antibodies are produced, how they interact with their targets, and how this process can be controlled or altered, is crucial for improving vaccination outcomes and generating effective therapeutic antibodies. The complexity of these biological processes, coupled with the need for precision in their manipulation, underscores the necessity for continued research and innovation. This level of complexity also makes this work impossible to do without using animals.

Our current therapeutic antibody discovery licence, whilst able to do some innovation, cannot investigate the basic biology of antibody generation. It does however sometimes produces data that highlights a possible improvement that could be investigated further. The purpose of this licence is to further understand how antibodies are produced, how they interact with their targets, and how this process can be controlled or altered to improve outcomes in terms of the quality of antibodies generated.
This may be done by:

- Testing new immunisation methods in comparison to existing one and demonstrating improvements.

- Producing an immune response to an antigen to generate immune cells that can then be experimented with to innovate/enhance our screening and characterisation work.

- The ultimate goal is to then to utilise this knowledge on our therapeutic antibody discovery licence, where the outcomes of this work will be applied to make better antibody medicines to treat patients.

What outputs do you think you will see at the end of this project?

We will have increased our knowledge and understanding of the processes for generating antibodies in mammals and how immunisation can be manipulated to produce better quality antibodies.
We will have informed the scientific community of the outcomes of this work by writing of thesis, presenting at scientific conferences, and possibly publishing journal articles.

Who or what will benefit from these outputs, and how?

This endeavor aims to enhance our comprehension of immune mechanisms, the production of antibodies, and the potential manipulation of the antibody generation process. Any advancements we uncover will subsequently be applied to the discovery of therapeutic antibodies, with the goal of creating more effective antibodies at a quicker pace for patients.

In the short term, the scientific community seem likely to benefit from new information on immune mechanisms, how antibodies are produced and how this process can be

manipulated. In the middle to longer term, patients may benefit from better therapeutic antibodies that work faster to help improve their treatment outcomes.
The scientific community will be kept informed of our progress and breakthroughs through conference presentations, patents filed and journal publications.

How will you look to maximise the outputs of this work?

If successful, this work would be used in new antibody discovery campaigns to enable our project teams to have the best chance of developing a new medicine for patients.
The work will be presented at national and international conferences to the scientific community.

Species and numbers of animals expected to be used

- Mice: 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.

We are using transgenic adult mice that are genetically altered to use human antibody genes and therefore make human antibodies that can be used to treat disease in humans. As we are aiming to develop new improved methods of immunisation to make better antibodies for treating human disease this mouse strain is the best choice.

Typically, what will be done to an animal used in your project?

Immunisation involves giving a substance to an animal usually by injection under the skin (subcutaneous), into the muscle (intramuscular), into the gut abdominal cavity (intraperitoneal) or via a vein (intravenous). The substance (antigen) can be a protein, genetic material, or a cell, and may be mixed with additives called ‘adjuvants’ to make it more likely to produce an immune response.

The mice will only experience a very short and slight discomfort and anaesthesia may be used for restraint purposes as required. This will need to be repeated as part of the process of generating an immune response.

Mice may be given immune modulator treatments either prior to the start of immunisation, at the same time of immunisation, or post immunisation by subcutaneous, intraperitoneal, or intravenous administration routes.

Once the immunised mice have generated suitable levels of antibodies, as confirmed by taking blood samples and testing in an assay, they will be humanely killed and their immune tissues (lymph nodes, spleens, etc) will be removed to enable us to isolate the antibody producing cells.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of immunisations will cause no more than transient mild pain to the mice. Post immunisation the mice can exhibit some general signs of being unwell: for example, they may show weight loss or changes in behaviour or posture.
When immune modulators are dosed, they are not expected to have any adverse effects of their own, and any adverse effects observed would be mild, transient, and associated with the dosing route as with immunisation.

Mice are expected to recover over a 48-hour period after immunisation or modulator treatment.

Rarely adverse effects that can occur are inflammation at the site of immunisation that can cause swelling or scab formation. In very rare cases this can lead to ulceration at the site of injection.

Very rarely, after the mice have been immunised, it may suffer an allergic reaction where mice will develop difficulty breathing. When this happens, the mice are immediately humanely killed to prevent any 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)?

Most adverse effects are expected to be mild but in 30% of cases there could be moderate adverse effects (typically this is post immunisation weight loss but can include ulceration at the site of injection (<5% of animals) and tumour growth from syngeneic cell immunisation (<1% of animals)

What will happen to animals used in 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 still many disease targets that non-animal methods fail to isolate antibodies for, but that can generate an antibody response in mice. However, this is still a difficult process in mice and requires further improvement to improve the robustness of the response and the quality of the antibodies generated.

To improve the status quo, we need to further understand the complex biology of how antibodies are generated in response to antigens. This process happens within multiple organs in the animals and with multiple cell types interacting, communicating, and relocating throughout the body. This complexity of this process is so great that there are no non-animal alternatives that can mimic the immune system in how it generates and develops an immune response.

Ex-vivo tissues can be used to attempt to replicate a single organ or tissue but this cannot replicate an immune response due to the multiple organs and time duration required for an immune response to develop.
Therefore, it is not possible to investigate and complete this work without the use of mice.

Which non-animal alternatives did you consider for use in this project?

We do have access to a non-animal antibody discovery platform (yeast display) but it cannot be used to increase our knowledge of the immune response in mice.

Why were they not suitable?

These are not suitable as they cannot mimic how mice generate and develop an immune response.

Non-animal methods are not capable of mimicking the process of antibody generation following immunisation and the subsequent maturing of that response. Also, it is impossible to investigate the factors affecting immunisation outcomes using a non-animal antibody discovery platform.

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 chosen based on a typical study design, that has been extensively used over many years, and animal numbers that are required to give significance to the output, multiplied by a projection of how many studies will occur over the 5-year period.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Design of experiment involving consultation/review by a statistician is used by default as part of the robust study design initiative when planning immunisation groups.
A statistician will also be part of the review panel for the in-vivo work to ensure that statistically significant observations can be made and compared for each animal group 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 historical immunisation data using our transgenic mouse line will be used to calculate the number of mice required for a study based on number of mice that have responded in the past. Additionally, we will use mice both sexes to ensure that there is minimal excess mouse production from 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 use immunisation methods to generate immune responses to our disease targets. Mice will be immunised using methods similar to human vaccination: injection of substances usually under the skin, but sometimes into the peritoneal cavity or by intravenous injection.

Our methods and reagents that we use are chosen with intention to have minimum impact on animal welfare during the immunisation period. The immunisation procedures will be of the shortest duration with the minimum number of injections (hence minimising any pain, suffering, distress, and lasting harm) required to generate the robust response required.

If during a study, adverse effects due to a specific reagent/technique are observed, discussions with subject matter experts on what options there are to either remove the reagent or change how it is used are held.

Where we judge the impact on animal welfare of using specific techniques or reagents is too great, these will be discontinued from the current and any future immunisations.

Why can’t you use animals that are less sentient?

It is necessary for the immune system in the animals to be working normally, and to ensure this, that the animal has achieved adult stage. Immunisation and the immune response are a process that can take weeks/months to mature and as such it is not possible to do with immature, less sentient or terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Prior to immunisation, all immunogens and substances are stringently checked for contaminants and sterility to ensure there is no potential for causing illness during the immunisation. Furthermore, any new substance(s) used are thoroughly researched for any potential adverse effects that could occur prior to being used.

Our immunisations have been refined to minimise any distress because of the immunisation process. Our examples of this are:

- Minimising pain during the immunisation by using fine gauge needles and using anaesthesia when multiple injections are required.
- When using substances to improve the immunisation process, we make sure these do not cause any additional suffering or distress on the mice by thoroughly researching them.

- Mice will be handled by non-aversive methods such as tunnel handling.
- We provide supportive care (e.g., provision of soft diet and warming) for any mice that are affected by immunisation to minimize suffering and help them recover as quickly as possible

Once we have achieved an optimal immune response, no further immunisations other than final boosting will be done.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The published principles and philosophies behind the PREPARE (2018) and original ARRIVE (2010) guidelines have been incorporated into our internal project planning standards of care and standard operating procedures. All work carried out under authority of this licence will undergo assessment of the study design during planning stages as part of a peer review process that is based on those guidelines, and will include statistical consultation. Facilities and processes are audited by independent bodies such as AAALAC International which has published guidelines and procedures to ensure work is carried out to high ethical and humane standards. The following published documents will advise on experimental design, animal welfare and husbandry during the life cycle of this licence:

- Kilkenny C et al (2010). Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. PLoS Biol 8(6).

- Smith A et al (2018). PREPARE: guidelines for planning animal research and testing. Lab Anim; 52(2):135-141.

- Percie du Sert N et al. The ARRIVE guidelines 2019: updated guidelines for reporting animal research. BioRxiv. 2019: 703181.

- NC3R's - Responsibility in the use of animals in bioscience research: expectations of the major research council and charitable funding bodies (2019).

- Guidance on the operation of the Animals (Scientific Procedures) Act 1986. (Home Office 2014).

- LASA - Guiding principles on good practice for animal welfare and ethical review bodies. (2015)

- Prescott MJ, Lidster K. Improving the quality of science through better animal welfare: the
- NC3Rs strategy. Lab Animal 46(4):152-156, (2017).

- Review of harm-benefit analysis in the use of animals in research. Report of the Animals in Science Committee Harm-Benefit Analysis Sub-Group chaired by Professor Gail Davies (Nov 2017).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I routinely receive NC3R's updates within the company that are sent to everyone involved in animal experimentation from the NIO.
Our AWERB meetings also highlight any 3R's news to PPL holders. Any that a relevant to this project work will be considered and implemented.
I also receive email updates from EARA on the latest standards and improvements in animal welfare.

Our research department uses non-animal alternatives for antibody discovery where possible to deliver therapeutic antibodies. This makes me very much aware of the capabilities and successes in utilizing

in-vitro methods.
Conference attending/speaking to learn from other experts in the field.

87. How do leukaemia cells interact with their microenvironment?

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

leukaemia, microenvironment, stromal cells, therapy, patient-derived xenografts

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?

Cancer (leukaemia) cells do not survive on their own. They are adept at subverting normal cells around them to provide support. Our lab aims to understand how leukaemia cells interact with neighbouring cells in the bone marrow with the overall goal of interrupting those interactions for the benefit of patients with 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?

The proposed murine work is part of a larger, national, collaborative project to improve the outcome for adult patients with a particular subtype of leukaemia named acute lymphoblastic leukaemia, which, although often curable in young persons, has a very poor outcome in adults and is incurable in older adult . These clinical trials which are accompanied by ‘correlative science’ aims and questions.
Many of the scientific studies arising from these trials use specimens which were donated by patients participating in our trials. This allows us to study the relationship between

genes and proteins expressed by the cancer cells AND to understand the relationships between the cancer cells and surrounding normal cells. We can directly relate our lab findings to the clinical outcome data that we have gathered, if we use patient-derived material.

We need to use mice in some of these studies for several reasons - first, to generate 'patient-derived xenografts' (PDX) namely, to amplify the patient leukaemia cells by allowing them to grow and multiply in mice. These cells do not multiply at all outside of mammalian hosts - using mice to propogate them means that we can make the most of cells that patients have donated which would otherwise be a very limited resource. We also need to use mouse models to see how these cells interact with the surrounding normal tissues and to test possible new approaches to treatment that we generate from our research and the research of our collaborators. Although we can do some of this work on the laboratory bench there are some aspects that cannot be studied outside of a mammalian body.

What outputs do you think you will see at the end of this project?

We will generate more patient-derived xenografts (PDX) representing the variety of genetic subtypes of acute lymphoblastic leukemia (ALL) which will make the most of the cancer cells that patients have kindly donated after participating in trials. These can be used in our experiments and will also be shared with other researchers. (Objective 1)
We will generate new information about how ALL cell interact with their surrounding tissues which we will share with other researchers via talks, conference presentations and publications in the scientific literature. (objective 2)

We hope to identify new therapeutic strategies that can be tested in clinical trials. (objective 3)

Who or what will benefit from these outputs, and how?

In the short term - from an immediate start until the end of the project- the host lab and wider collaborators will benefit from what we do. Within the host lab, MSc, PhD students and post-doctoral researchers will be able to conduct the necessary experiments to complete their projects.
The wider collaborative team across the UK will benefit from data that we generate and from patient- derived xenografts that we create.
The University employers of the host lab - will benefit if our work is successful. The wider research community will benefit from any data that we publish.
In the longer term 5-10 years, any new approaches that we identify may benefit patients with ALL.

How will you look to maximise the outputs of this work?

We collaborate widely already and will continue to do so. We are experienced in the dissemination of new knowledge by submissions to peer-reviewed journals and presentations at conferences.

Species and numbers of animals expected to be used

- Mice: We have provided the maximum estimate of 1000 immunocompromised mice for the lifetime of this project.

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 6-12 week old immunodeficient mouse strains. We choose younger animals so that the immunodeficient state is maintained as well as possible throughout the experiment. We need to use immunodeficient mice as we are giving them human cells to generate a model of human leukaemia.

Typically, what will be done to an animal used in your project?

Healthy mice are injected with human leukaemia cells via the tail vein. We sometimes need to check if the leukaemia has engrafted by blood sampling or occasional sampling of the bone marrow. If we are trying to propagate primary human leukaemia specimens, the mice sometimes need to be irradiated prior to the injection. We may inject portions of the same human sample into multiple separate mice, usually 3. After irradiation, the mice sometimes lose weight, but they usually recover well after about a week.

During this time, they receive additional monitoring and care including softer diet and sometimes, antibiotics in their drinking water. On occasion, they may receive subcutaneous fluid (normal saline, dextrose saline or other appropriate fluid) both proactively and if they are not drinking adequately. It may take many weeks for engraftment to occur and during this time, the mice are left to their normal environment and behaviours, they may have occasional blood samples taken to see if the leukaemia has engrafted. When engraftment is confirmed, we euthanise and harvest the cells from the mice. If we are using the mice engrafted with leukaemia for experiments, rather than just to propagate the leukaemia cells, they may receive injections, usually via the tail vein but sometimes via the peritoneal route of various treatments (eg chemotherapy, antibodies, small molecules) as potential treatments that we are evaluating. When we are doing 'therapeutic' experiments with cell line the duration is usually about 3-6 weeks before mice reach the humane end points. If the experiment is with patient-derived xenografts (PDX) which are tumor cells taken directly from a patient, they are not so aggressive in generating leukaemia, so the mice humane endpoint may take 10-12 weeks. NB A longer time to reach humane endpoint does not mean more suffering - means that it takes longer for the mice to begin to develop signs of leukaemia.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice may experience transient, mild pain during injections. Mice that have received irradiation may have a temporary diminution (1 week) in appetite and well-being including an increased risk of infections - they are provided with a soft diet and sometimes antibiotics may be added to their drinking water. Mice will develop leukaemia, which is a disseminated disease, so there is no visible tumour. For 3-4 weeks they usually do not suffer any adverse consequences and there is no impact on their behaviour. Of course, the leukaemia will eventually begin to impact on their well-being toward the end of the experiment - this is then the humane end-point. We monitor the mice daily and use a scoring system for the humane endpoint which includes monitoring their oral intake, their grooming behaviours, their posture and interactions with each other, and their weight. We

are very used to also looking for other indications of concern e.g. hind limb paralysis which can be a sign of leukaemia which we know often means they are nearing the humane end-point. We take into consideration the possibility that where males are housed together littermates may succumb and a single male mouse may be left alone. This is an unusual occurrence and these mice will be kept for the shortest possible time in order to collect the required data (for example in an endpoint therapeutic experiment) . We can minimise this by housing the same male littermate mice that have received the same approach in the same cages so that they are more likely to reach the endpoint at the same time.

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 of our protocols are considered 'moderate' - hence all animals may experience moderate severity

What will happen to animals used in 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?

Presently, animals are the only models in which the behaviour of primary patient acute lymphoblastic leukaemia cells sufficiently closely resembles that in human bone marrow that it is acceptable for certain types of experiments. As an example, primary patient material does not proliferate in ex-vivo models. Additionally, approaches which are testing potential novel therapies still rely on animal models to provide data which is acceptable to the regulatory authorities. Ex vivo models, whilst they are in active development, are not presently suitable for any of the work above. A whole mammalian body is required to study the proliferation of the disease.

Which non-animal alternatives did you consider for use in this project?

We are actively working on several lab approaches to avoid the use of mouse models.

- The first is the use of a human bone marrow organoid, in collaboration with a colleague in a different university. For the first time, primary ALL cells were shown to proliferate in this context. Several members of the applicant's lab are now working to adapt this organoid to our specific uses. However, this is ongoing work and at present does not obviate the need for some murine work.

- The second is to explore an ex-vivo, cytokine-free haematopoietic stem cell culture system (this is a system which has been used to expand mouse and human bone marrow stem cells outside the body) to see if it will provide the possibility to expand primary ALL material. This is an ongoing collaboration between the applicant’s lab and lab in the same department but the work is at an early stage.

- PDX models, once developed, can help somewhat with avoiding murine experiments but at present they still need passaging in mice to replenish the cells as PDX do not grow outside a mammalian body. Approaches 1. and 2. above may help obviate the need for that.

Why were they not suitable?

As explained above, it may never be possible to completely exclude animal models for the purposes of generating preclinical data evaluating novel therapeutic approaches, but we have a genuine hope to reduce and refine murine experiments within the life of this PPL, using the approaches 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?

Where we are using mouse tissues for ex vivo endpoints - an example might be harvesting murine mesenchymal stromal cells (MSC) which have been exposed to human leukaemia cells - for use in an ex vivo experiment, the number of mice are guided by the number of cells that are needed for that experiment e.g. flow cytometry and for the requisite experimental repeats. Often, this means we may need 3-4 mice per group.

Our SOPs and data analyses are continuously optimised to use the fewest number of animals possible. Where we are doing survival analyses after responses to treatments, we perform power calculations, based on the anticipated effect size, in order to determine group sizes. The overall estimate given takes into account the possibility of doing experiments that we cannot currently foresee. We follow ARRIVE guidelines for the reporting of our animal experiments in publications.

We used the NC3Rs experimental design assistant to assist with total number planning

A total mouse number of 1000 has been arrived at to take into account the maximum possible mice we would wish to use - this estimate is based on actual animal use in our prior PPLs and takes into account that the lab is a relatively infrequent user of mouse models. For each individual experiment, we make specific calculations as above.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

This application does not underpin our entire lab program – our work is largely carried out in vitro, so we have only directly planned out the subset of experiments that we are currently funded to do. It is important to note that we seek to minimise the use of mice overall but that we don’t always seek to minimise the number per experiment.

Numbers of mice used will also be reduced by responsible breeding practices of GA animals according to published best practice.

The experiments that we conduct need to have sufficient power to detect the end point being studied with appropriate statistical significance but within the experiment we seek to use the minimum number of animals to provide sufficient power. We used on-line power calculators for the current plan but we are always willing and able to seek statistical advice where we are unclear.

We will always publish negative results according to ARRIVE guidelines recommendations to ensure other workers do not need to recapitulate what we have done.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Most importantly, we seek to minimise the number of mice used by not doing experiments with mice at all. We prefer to use human cells and tissues. Where mice are a pre-requisite, we use efficient breeding by using the institutional breeding protocols. We conduct pilot studies with small numbers of mice to ensure we understand effect sizes and before planning larger studies so that we can optimise study design. We share tissues with other researchers wherever possible. We use the PREPARE guidelines to help us design and manage our work.
We will publish according to ARRIVE 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.

We use immunodeficient mice so that human leukaemia cells are able to engraft and we can study 1. the relationship between the bone marrow and the leukaemia cells 2. evaluate possible treatments 3. amplify small numbers of leukaemia cells kindly donated by patients in clinical trials so that we can make the best of those samples as they are a non-replenishable resource. We choose techniques which are least painful to the mice and always use analgesia and anaesthesia where needed. We have a very thorough scoring system to determine the humane end point. We have a lot of experience of taking care of mice in these sorts of experiments.

Why can’t you use animals that are less sentient?

mice are the 'lowest' species in which this work is possible - this work would not be possible without mice - the tumor cells we work with only survive in a mammalian host

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We very regularly monitor the mice under our care especially as they reach the humane endpoint. The frequency of monitoring is determined by the type of experiment and our observations of the mice.

Once the mice have developed leukaemia and/or received therapy, they are monitored daily. We ask for assistance from the facility staff if we are concerned. We take advice from and cooperate closely with facility staff to make sure the animals are housed and cared for properly. We use anaesthesia and analgesia to minimise pain where necessary.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I find the NC3Rs website extremely informative. We are also strongly guided by advice from our NACWOs, staff in the BSF in general and if necessary, discussions with NVS. We work to PREPARE, ARRIVE, BJ of Cancer, Workman Guidelines and LASA and other guidelines - all mentioned elsewhere in this application.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am a member of my host institution's bioservices facility (BSF) advisory committee, so I am fortunate to participate in updates and discussions about progress and changes in 3Rs approaches. Additionally, I receive and read all new information initiatives circulated by our BSF. I am planning to submit a NC3Rs grant application regarding the bone marrow organoid model - the NC3Rs website is extremely informative and helpful. I attend scientific conferences, read the scientific literature and interact with colleagues to be aware of new models we might use instead of mice. My host institution runs a BSF forum meeting several times per year where all PPL and PIL holders are invited to review BSF operations and where BSF staff can pass on new developments.

88. Mechanisms regulating the formation, function and stability of neuronal connections in the vertebrate 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
  - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Synaptic signalling molecules, Synaptic degeneration, Neuronal connectivity, Neurodegenerative disorders, Alzheimer's disease

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 the function of signalling proteins that regulate the formation, function, and maintenance of connections between nerve cells, also named neurons, in the healthy brain. We are also interested in understanding how neuronal circuits degenerate in pathological conditions such as Alzheimer’s disease (AD).

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 cellular and molecular mechanisms that control the formation, function and stability of neuronal connections will help to identify how these become dysfunctional and degenerate in neurological conditions such as Alzheimer's disease. This research will lead to the development of new therapeutic strategies for the treatment of these conditions.

What outputs do you think you will see at the end of this project?

We aim to shed new light onto the mechanisms that regulate the formation, function and stability of neuronal connections, also called synapses. We hope that our research will lead to the discovery of new targets that can be modulated to promote synaptic connectivity in the healthy and diseased brain. Findings from our studies will be published as research articles or reviews in international scientific journals. We will also report our results to funders and will give presentations of our results at national and international scientific meetings. Through outreaching activities, we will also communicate our results to the general public. In addition, we plan to continue our collaborations with companies to develop drugs that could be used as therapeutic approaches to treat neurological conditions in the future.

Who or what will benefit from these outputs, and how?

In the short term, our results will benefit other scientists working in the fields of neuronal connectivity and neurodegenerative diseases. In the long term, they will provide crucial information for developing new therapeutic approaches to protect synaptic connections in diseases such as AD and therefore ameliorate or cure these conditions. Despite extensive research, there is no effective treatment for people suffering from memory loss in AD. Our research could change this by identifying new molecular targets that would be used to protect and restore synaptic connections in humans suffering from neurodegenerative diseases.

How will you look to maximise the outputs of this work?

In general, animals will be examined using different approaches before we conduct behavioural tests. Therefore, we will have obtained considerable information about the phenotype of each animal. We will use the most advanced techniques available for monitoring and manipulating gene expression that could result in changes in neuronal connectivity. The use of several different types of approaches will maximise outputs and will minimise the number of animals used.

In addition, most data, when possible, will be acquired and analysed automatically with custom software. This software acquires all cellular, biochemical, and behaviourally relevant information during the experiment, and this data will be logged to provide a substrate for further analysis and for planning further experiments later. When possible, we will share our data once the study is complete so that other scientists learn from our studies and better plan their experiments.

Raw data and analysis methods will be shared with the scientific community (following peer-reviewed publication), to allow other scientists to gain insights from our experiments, plan better experiments and reduce replication of work.
Sharing will be done through scientific publications, reports, and presentations at scientific meetings.

Species and numbers of animals expected to be used

- Mice: 15,800

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.

Extensive research has been conducted in rodents (mice and rats) to determine the cellular and molecular mechanisms that regulate neuronal circuit formation and function. Both during early life, and at later stages including adult and aging animals. Early life stages are often most useful when studying circuit formation, whereas aging animals are often necessary when investigating mechanisms in conditions like Alzheimer's disease (AD). In addition, many mouse models carrying specific gene mutations have been generated for their use in scientific research. For instance, several mouse models carrying mutations linked to AD have been generated and are available to researchers around the world. Some of these mice will be used in our research to determine if we can ameliorate synapse loss and dysfunction by experimental manipulation. We aim to identify molecules that could be used for the treatment of diseases such as AD.

Typically, what will be done to an animal used in your project?

We will generate, obtain from our labs or buy mice from standard suppliers, breed them at our facility or import them from other labs in the UK, Europe or other countries. Some of these mice, that are genetically modified, will be bred in our facilities to generate the desirable genotype to characterise how certain changes in genes affect the connections between brain cells. We are particularly interested in how connections between nerve cells are formed and maintained, and their function in the developing, adult, and ageing mouse brain. In some cases, we will manipulate specific genes by turning them on or off in the brain. We will use knockout mice in which the mouse lacks specific genes, inducible transgenic mouse models, or viral infections to control when (specific time points) and where (specific brain regions, such as the hippocampus) our target genes are active in the brain.

To evaluate changes at the synapse (specific connections between nerve cells), we will isolate different types of brain cells from mice or rats, including nerve cells (neurons) and other cell types that support the function of neurons (glia). These different populations of cells will be maintained for several days or weeks in a dish and subjected to manipulations, such as drug treatments. Alternatively, we will also obtain brain slices and other tissues from mice that could be fixed for following analyses. These biological samples will be used to evaluate structural and functional changes at synapses using molecular and biochemical methods, including analyses of gene expression and protein levels. We will also use cellular techniques (such as live imaging and immunofluorescence microscopy) and electrophysiology to assess the function of synapses. To evaluate the whole circuit function, we will use behavioural studies to test, for example, changes in learning and memory in live animals.

Animals will be kept for different periods of time depending on the scientific question. For analyses of synapse formation, neuronal and glial cells will be isolated at early embryonic or postnatal stages and cultured in a dish for several days or weeks. For analyses in the

mouse brain, we will use young animals (for example, at postnatal day 15). In other studies, we will use juvenile - adult mice (from postnatal day 21 up to 15 months of age, but typically up to 4-9 months of age). To study ageing, we will use mice from 15 to 20 months.

For injections of viruses or substances to manipulate the expression of specific genes, we will use mice of a range of ages. For postnatal studies, viruses will be injected into the ventricles of the brain of new-born mice between 0 to 5 days of age and kept typically until 15-21 days. For adult mice, we will inject viruses or substances from postnatal day 6 (although typically from 2 months of age) using stereotactic injections into the brain. For ageing mice, we will inject at an age between 1 to 15 months and age up to 20 months or inject the animals after 15 months of age and keep up to 20 months old.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect significant adverse effects for any of our animals due to their genotype or experimental interventions.
Based on our experience, we predict that our mice will not exhibit adverse effects such as pain, formation of tumours or abnormal behaviour.
Our research is focused on studying early postnatal stages and adult mice with no adverse effects. In addition, our research in AD requires the use of ageing mice (up to 20 months, which is within the average life span for mice) and therefore, protocols for the use of ageing mice are included in this licence.

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:
Mild severity 70-50%
Moderate severity 25-40%
Subthreshold 5-10%

What will happen to animals used in 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 overarching aim is to elucidate the mechanisms that regulate the formation and function of synaptic connections in the vertebrate brain and how the structure and function of synapses are maintained in the healthy and diseased brain. Given that no cell line models are available to study neuronal connectivity, the use of animals is crucial to

understand how neurons connect with one another and how their interactions contribute to high functions such as memory. Unfortunately, there is no other system that can replace animals for our studies.

Which non-animal alternatives did you consider for use in this project?

For certain types of experiments, we might use cell lines to express constructs to validate, for example, protein interactions or the signalling pathways involved. This information will be crucial to narrow down what experiments will be done using animals. Similarly, we might use neurons derived from human induced pluripotent stem cells (iPSCs), but they have serious limitations for the questions we want to address, as differentiated neurons from human or rodent iPSCs do not form functional synapses when using current protocols. However, it is possible that in the future, new protocols will be available to obtain the maturation of neurons that form functional networks.
Once we have established certain principles using cultured neurons/glial cells, we will use animals to study neuronal connectivity including complex functions such as learning and memory.

Why were they not suitable?

No cell lines, even neuronal cell lines, make synapses when cultured isolated in a dish (in vitro).
Therefore, many of our questions on neuronal connectivity could not be addressed using this system. Unfortunately, neurons derived from human or rodent iPSCs do not reach sufficient maturity in culture to evaluate the formation or function of synapses. Thus, studying neuronal connectivity is not possible using iPSCs using current protocols. As our research aims to study the impact of molecules/genes on the function of complex neuronal connections that control functions such as memory, the only available model system is the intact 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?

These numbers included here are based on our extensive experience (27-30 years) of working with rodents to generate statistically robust data. In addition, these numbers are based on observations and discussions with other laboratories undertaking similar studies. They are also based on the evaluation of published studies like ours, and by the input of reviewers to our grant applications and research articles in scientific journals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our first step is to determine if we could address some of the questions using cell lines (although limited for our studies). These studies could provide, for example, information of protein-protein interactions or protein localisation. Having this information, we will move to

cultures of primary neurons and/or glial cells to determine gene function. The next step is to perform studies using animals and determine changes in synapse function and structure in the intact animal. If we have evidence for a role of a gene/protein in neuronal connectivity, we will then perform behavioural studies to examine the whole circuit function (learning and memory).
To ensure we obtain meaningful results with the minimal use of number of animals, we use appropriate and standardized mathematical strategies (power calculations). For this, we use our previous experimental design experience, bearing in mind the type of analyses we will perform to reliably obtain robust data. By following these steps, we will ensure that we use the minimum but necessary number of animals for our experiments to reach statistically significant differences.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Sex: The phenotype of our mice could be affected by sex. For example, female mice, which regularly undergo the oestrous cycle, experience natural fluctuations in their circulating levels of sex hormones and therefore affect the function and number of synapses. When possible, we will use both sexes to determine possible differences in the phenotype.

Breeding: We have extensive experience in the breeding of our mouse lines, which allows us to determine the minimal number of breeding pairs necessary for our studies. If a new mouse line is used, we will follow advice from the sender (scientists or mouse providers such as the Jackson lab). We will follow their recommendations (https://www.jax.org/jax-mice-andservices/ customer-support/technical-support/breeding-and-husbandry-support/colony-planning). We will also bring in new parent strains on which our mouse models were founded, to regularly backcross colonies to ensure good genetic penetrance of the mutation and avoid inbreeding. This will provide healthier and larger litters and therefore reduce the number of breeding pairs.

Experimental bias: To improve our statistical robustness, improve randomization, and reduce our experimental bias, we will conduct blind experiments when possible. The experimentalist will be blinded to the experimental condition or genotype of the animals (e.g. control or wild type versus mutant or transgenic mice). For this, a lab member will blindly label the samples that will be evaluated
(for example, using microscopy or biochemical approaches) or randomise the animals (for electrophysiology). For behavioural analyses, animals will be randomised, and the operator will be blinded to the genotype or conditions throughout the protocol including data analysis.

Drug dosing: Animals will be exposed to a drug to see its impact on neuronal connectivity and whether the drug can restore memory. The dose will depend on prior use of the drug in a similar context (performed by us or other scientists) and considering the phenotype and age of the animals. If data from the literature is not available, we will use ex-vivo approaches (brain slices from the relevant mice and expose them to different drug concentrations). This information will be used to estimate the dose in vivo. This approach will avoid the in vivo use of several doses and a large number of animals.

Cryopreservation: We currently have 6 mouse models cryopreserved. This provides many benefits (as stated by NC3Rs) and allows us to be flexible in keeping the minimal

number of necessary colonies for our studies. In addition, cryopreservation allows us to share these strains with colleagues in the same research field.

Genotyping: We undertake this for nearly 80-90% of our mouse colonies (parents and litters) to reduce unnecessary breeding, allow experimental blinding, and reduce genetic drift. Genotyping will be done as soon as animals are weaned (or earlier if possible) to reduce the maintenance of unwanted animals and to run a more efficient breeding program.

Tissue sharing: We routinely share animal tissues between members of our laboratory and those in other local laboratories or our collaborators.

Power calculations: Where necessary, we use power calculations to estimate how many animals are required to ensure that the results observed are statistically robust. These calculations are usually performed using G*Power (https://stats.oarc.ucla.edu/other/gpower/).

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 combination of control mice (wildtype), which do not carry any mutations or inserted genes, transgenic mice expressing target genes, knockout mice in which the target gene has been deleted, and knock-in mice carrying mutations of interest. For example, we will use mouse models of AD such as NLGF, and inducible transgenic mouse models.

Why can’t you use animals that are less sentient?

We study the mechanisms that control the formation, function, and stability of connections between nerve cells (neurons). In addition, we aim to understand how these mechanisms contribute to complex animal behaviours involving learning and memory. For this, we need to use animals from later postnatal stages and adult mice. We are also interested in understanding the role of ageing in the function and stability of neuronal connections as occurs in conditions such as Alzheimer’s disease. For this, we need to use ageing mice up to the age of 20 months.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

There are several approaches that we can take to refine our procedures.

Behaviour: Pre-experimental handling habituation will be used as it significantly reduces the stress animals experience. We regularly review our procedures among ourselves and

liaise with colleagues and BSU staff to improve this. Occasionally, we might require to singly house animals for certain behavioural tests which might require up to five days of habituation followed by testing for up to two weeks. Where appropriate, we will pre-expose an animal to other behavioural apparatus (such as the elevated plus maze), to ensure that this initial stressor has minimal impact. We also follow updates from the 3Rs Group to consider the best test to address our scientific question.

Surgeries: Surgical procedures are scrutinised by the BSU staff prior to implementation, for perioperative care and pain management, and the associated record keeping. We therefore adhere to a very proactive and animal-centric welfare regime. All surgical techniques are carried out in anaesthetised animals according to the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017). All animals are expected to have a rapid and unremarkable recovery from the anaesthesia. Then they will be returned to their home cages when they are fully recovered. In the uncommon event of failing to do so, or presenting any signs of distress and pain that cannot be remedied promptly following NACWO and/or NVS advice, animals will be humanely killed by a Schedule 1 method or perfusion fixation under terminal anaesthesia [AC]. If minimal post-operative complications or discomfort occur, enhanced monitoring and care will be provided until the animal fully recovers.
For viral injections into the brain, we always undertake a pilot study when using new variants (or novel injectable compounds). This is to ascertain optimal viral titers (injection volumes) and transduction (expression of genes/proteins) times, as every new virus has different efficiency. This means we will be able to optimise (and shorten) the latency between viral injections and phenotype assessment. Drug injection or cannula insertion will be performed under general anaesthesia during the procedure. In the case of pre-weaning animals, they will be returned to their home cage with their mother and siblings. For adults, animals will be returned to their home cages as soon as they are fully recovered from surgery.
We undergo regular training to hone our procedural skills, which reflects on much-reduced postoperative complications. This will minimise the timeline to the ultimate ‘use’ of the animal and its welfare costs. In all our previous Project licences (PPL), we have rarely experienced any complications in the welfare of animals under our care.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The BSU website publishes and updates regular ‘Policies and guidance’ notifications for BSU users to engage with for guidance on all aspects of animal welfare and experiment design.
We always seek advice from Local NVS and NACWO and BSU staff and relevant guiding principles for good practice. For example, LASA has an extensive selection of publications available for researchers: https://www.lasa.co.uk/current_publications/
Various organisations are proactive in publishing guidance on animal welfare issues, which we receive as part of regular electronic table of content (eToC) notifications relating to these topics. Equally, we continually make extensive use of the published literature in our field regarding experimental design and practice. We also use other resources hosted on the NC3Rs website such as "ARRIVE guidelines on experimental design and reporting results", "Procedures with Care", and "Aseptic Technique in Rodent Surgery'.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep in regular contact with our local NVS and NACWO to ensure we maintain best practices.
We will liaise with NC3Rs representatives to ensure we are up to date with current best practices.
We will use the resources published on the NC3Rs website to ensure that the group undergoes continuous training and professional development with respect to the 3Rs.
We will follow technological advances published in the scientific literature, allowing more efficient recording techniques. For example, yielding more data per animal, best virus serotype for efficient expression of genes or miniaturising recording equipment. We will also discuss with other scientists who are using similar techniques to ours so that we use the best approaches.