Home Office

Animals (Scientific Procedures) Act 1986

Non-technical summaries for project licences granted October - December 2023

Contents

1. Breeding and maintenance of wildtype and genetically modified mice to study effects on vascular function

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

pulmonary arterial hypertension, remodelling, therapy, cardiovascular, connexin

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

This licence aims to preserve and use a valuable colony of genetically altered mice that have reduced expression of a gene known to play important roles in vascular 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?

Cardiovascular diseases, or diseases which indirectly affect the cardiovascular system have a huge impact on our health. A greater understanding of the cardiovacular system will aid in developing better drugs with which to treat cardiovascular diseases. This project

will focus on diseases of the pulmonary vasculature such as pulmonary hypertension (PH). PH is a devastating disease with a poor prognosis and novel therapies are needed with which to treat this disease. Vascular and cardiac tissues from these mice can be used in in-vitro experiments to study the roles of a specific gene/ gene product in regulation of cardiovascular function and, more specifically, in the pulmonary vasculature.

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

The primary aim of this project is to preserve a colony of genetically altered mice which have already proved very valuable in elucidating the roles of a gene and its products in the heart, blood vessels and blood pressure (vascular function) and, specifically on the development of pulmonary hypertension.

These mice are (to the best of our knowledge) the only colony in the UK with an alteration in this specific gene of interest. Thus the primary output from this licence would be to save this research resource and continue using it. In the short term, tissues from the mice will be utilised to generate new information on the physiology of the pulmonary vasculature. In the medium and long term, the colony of mice will be transfered to our new licence - once ready - which will allow us (and our collaborators) to continue our investigations into the roles of the gene of interest in vascular function and the development of pulmonary hypertension. This information will be shared via conference presentations and publications.

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

In the short term, the mice generated from this project will be available for further scientific research (under a new Home Office licence, as explained above) and to be made available to the scientific community. Tissues gathered from these mice would generate new information on the regulation of the pulmonary vasculature.

In the longer term, preservation of this colony will lead to a thorough investigaton (under a new Home Office licence) on the pathophysiology of pulmonary hypertension. This knowledge will be useful to research scientists, clinicians and industry. If these outputs inform new treatments for PH, patients will also benefit from this study in the longer term.

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

We will collaborate and share tissues from these mice with groups working on various diseases. For example, we routinely share brain tissue, pancreas, eyes, testes and heart tissue from these mice with collaborators. Knowledge will be disseminated widely at national and international conferences and through peer-reviewed original research articles.

Species and numbers of animals expected to be used

- Mice: 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.

These mice have a genetic alteration which mean they have reduced amounts of a specific protein in all tissues. They can therefore be used to study the role of this protein in various tissue types and at different life stages. This project specifically aims to investigate the role of this protein in the pulmonary vasculature.

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

Wildtype and genetically modified mice will be bred and maintained in this project using standard practices. Typically, mice will have a tiny amount of tissue removed by ear notching to allow genotyping. Mice will be culled at different ages via humane Schedule 1 methods and tissues such as lung, heart and blood vessels removed for in-vitro experiments.

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

This project is for breeding and maintenance of mice only and no adverse effects are expected. We breed the strain as heterozygotes (i.e., animals have a “normal” copy of the gene of interest) which means that our mice don’t actually show any adverse effects. In the unlikely event of an unexpected adverse effect, advice will be sought from the Home Office and Named Persons (NACWO/NVS) or the animal will be humanely killed by a Schedule 1 method.

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

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

- Mild (100%)

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

- Killed
- Used in other projects

Replacement

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

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

This licence will allow preservation of a colony of transgenic mice. These mice will be used under a future licence (currently being written) to examine the development of PH. As this

is a complex disease involving interaction between all cells of the pulmonary vasculature, the immune system, hormonal mediators and cardiac cells, animal models are required. While it is possible to cryo-preserve mice, this technique is not always successful (i.e., there's always a risk of losing the strain). Moreover, we have a continuing need of tissues in order to perform our ongoing in-vitro experiments. We also have funding in place to carry out in vivo experiments using this colony of mice and plan to do so as soon as we have a continuation licence in place to cover these experiments. In the meantime, tissues will be taken from euthanised mice under this licence and results of these in vitro experiments will inform future in vivo experiments. Therefore, we think the best course of action to preserve the colony is to continue breeding the mice.

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

We already, to a large extent, use non-animal alternatives and will continue to do so. Much of our preliminary work is performed in cultured cells- often human cells purchased form approved sources. We also use historical samples of animal tissues, fluids and histological slides to minimise our use of animals. We also keep abreast with the literature in the field and have searched appropriate databases (e.g., AltWEB).

Why were they not suitable?

PH is a complex disease involving a dynamic interaction between the blood vessels in our lungs, the heart and the immune system; at present this complex interaction can only be examined in a whole animal model.

Reduction

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

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

The number has been estimated based on the fact that three breeding pairs are typically required to maintain the colony and to provide a steady supply of animals of the correct genotype for tissue harvest. We know from experience that this will generate approximately ten - twelve mice per month. This would generate approximately 300 mice over a two year period. However, litters may be larger than estimated and therefore we have requested a maximum of 400 mice to be allowed for the two year duration of the licence. When possible, we will seperate breeding pairs in order to keep the number in the colony low and thus avoid wastage.

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

The primary aim of this project is the preservation of a colony of GA mice. Numbers will be kept as low as possible whilst keeping enough mice to maintain the colony. Tissue will be harvested from mice and either used immediately or preserved and stored for future use.

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 harvest, use and preserve tissue from the animals, some for on-going in-vitro work, some for new pilot studies and some to share amongst other projects and researchers. As well as the heart and lung samples required for our own research, tissue samples such as brain, eyes, testes and pancreas have been harvested from these mice and shared with researchers working on a variety of conditions such as Alzheimer's and diabetes. It is our intention to continue sharing tissue.

Refinement

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

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

Transgenic mice will be bred in this project. Breeding, colony management and handling of mice will be done in accordance with the latest advice published on the NC3Rs website (nc3rs.org.uk)

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

When investigating the role of a gene or gene product on cardiovascular disease, genetically modified mice are the gold standard animal model to use. Multiple knock-out and knock-in mouse models exist which have been helping the scientific community to elucidate many different pathologies. Genetically, mice are very similar to humans increasing translational value of results compared to conducting this research in a less sentient species.

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

We wil ensure our breeders are at peak age for mating, that they do not have too many litters and that genotyping methods are the mildest possible.

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

We will follow the latest advice published on the NC3Rs website under 'Breeding and colony management', 'Handling and training of mice and rats for low stress procedures' and 'Housing and husbandry: mice'

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.org.uk). Our institution has a 3Rs newsletter and an annual 3Rs day involving presentations and workshops which the research team will attend. Our NIO also helps us to keep abreast of advances in the 3Rs and the NACWO and NVS assist us with implementing these advances where applicable. I'm also personally involved in our institution's 3Rs Focus Group.

2. Creation, breeding and maintenance of genetically altered rodents

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

Service support, GA breeding, Transgenic

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

The main aim of this licence is to provide a comprehensive service to researchers. The procedures and protocols that constitutes the PPL will result in genetically altered animals (GAA) being made available for use in a range of project licences involved in medical research.

This licence will support other PPL’s at the home establishment which have already been granted and have undergone a harm benefit analysis, accepted by the Secretary of State. The main objectives are:

To create novel GA models for use on other PPLs at the home establishment.
To breed and maintain GA animals for use on other PPLs at the home establishment or during a PPL renewal.

Cryopreservation of embryos and sperm from GA animals.

Reconstitution of GA lines for use on other PPLs at the home establishment.

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

Why is it important to undertake this work?

GAAs are an invaluable tool for understanding disease processes in man and animals and for the developing treatments and therapies for them. Several approaches exist to produce new GA models for disease research. These mainly rely on the ability to manipulate embryos at an early stage of development and then successfully produce offspring from those manipulated embryos. My team can provide the skills, knowledge and specialist equipment required to efficiently produce animals carrying specific genetic alterations with minimum animal wastage. Archiving of GA lines as frozen embryos and/or sperm from modified lines not only helps reduce animal use by minimising the number of GA lines maintained on the shelf, but also facilitates the sharing of GA lines between researchers, providing further opportunities for reduction. Repositorie's will be available for future use to safe guard against the potential loss of a line which would be difficult to replace.

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

The main function / outputs of this service licence is twofold. Firstly to breed and maintain GA animals and secondly to provide transgenic (TG) services.

The use of this licence is dependent on the demands from the research at the home establishment. The previous licence has been utilised extensively to lead the research teams towards gaining efficient provision of healthy animals for their research projects. Specifically, we have bred and maintained 58 mouse lines, cryopreserved 24 lines using sperm and embryo, completed 5 embryo rederivation projects and 8 invitro fertilization (IVF) projects. Therefore we believe the demand is there to support the need for this new licence.

In addition, in terms of breeding and maintenance, the previous licence has been particularly useful as a bridging licence for the breeding and maintenance of GA strains and has assisted 9 active PPLh during their applications for continuation PPLs.

The last PPL assisted researchers in the areas of Psychology, Medicine, Biosciences and Optometry.

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

The research community at the home establishment and members of local collaborating establishments will benefit from the use of this service project licence.

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

Once new lines are generated they will be transferred to the researcher to perform all the required experiments as outlined in their PPL. Their results will be published accordingly. Our tissue / animal sharing platform will be engaged to maximise the use of animals bred under this licence.

Species and numbers of animals expected to be used

- Mice: 27700
- Rats: 1500

Predicted harms

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

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

Mice are the most common and least sentient of the mammalian species used in scientific research. It is possible to alter mouse genes and manipulate the mouse genome to produce models for human diseases or conditions. The extesive range of established disease models and the fact that they share many common physiological processes with humans makes them the species of choice for many bio- medical research projects. Rats are less commonly used, but are the species of choice for studies that require a physically larger animal or those with a greater ability to learn and problem solve. The genetic manipulation of rats is more difficult than mice, however several important GA and harmful mutant rat lines exist that are of great value to many bio-medical research projects.

The animals used under this licence will be adults. The offspring produced may be used at any stage in the development, depending upon the scientific need.

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

Animals will be born, they will have an ear clip taken for identification or genotyping and then socially housed in a cage with environmental enrichment. Animals of suitable genotype, age and sex may be transferred to another licence within the establishment or to collaborators.

Sperm, eggs and embryos may be harvested after humane killing for rederivation or cryopreservation projects.

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

When new mouse lines are generated, founders carrying genetic modifications will be closely monitored for unexpected adverse effects.

After surgeries, animals will be closely monitored to assess their recovery. Analgesia will be provided before and after the surgery as required. Within these standard surgical procedures, it is estimated that any type of discomfort will be transient.

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

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

Based on Returns over the last four years we anticipate: Sub-threshold 60%, mild 34%. The remaining animals may experience moderate e.g following surgery 2% and severe e.g occasional animals are found dead 4%.

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

- Killed
- Used in other projects

Replacement

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

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

As there are no non-animal alternatives, the use of rodent models is needed to breed strains which show genotypes and/or phenotypes typical of human disease to help develop new novel therapeutics for the treatment of human disease.
Our local Animal Welfare and Ethical review Body will consider the harm/benefit ratio for all requests for new GAA models to be created, request for breeding and requests to cross established stains. The applications will request details of scientific background, objectives and the potential benefits of the project, to ensure the most appropriate animals are delivered to assist the applicant in meeting their objectives. The TG technologist will also make this assessment for each new TG project and advises on the most suitable approach.

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

Many of the research projects will involve the use of in-vitro systems such as cell culture, human tissue assays, computer modelling to complement the animal work.

Why were they not suitable?

- NA

Reduction

Explain how the numbers of animals for this project were determined.
Describe steps that have been taken to reduce animal numbers, and principles used to design studies.

Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 is based on usage over the previous five year period. This along with the experience of supplying GA rodents under previous service PPLs has given insight into future demands.

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

Long standing experience and the use of proven protocols for supply of GA rodents under previous PPL has given insight into optimal numbers required to maintain colonies and ensure minimal requirements.

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 completed in accordance with best practice by experienced technicians and the breeding programme subject to regular review to determine production targets, assess customer demand and to monitor and reduce any wastage. It is also important that the genetic integrity and provenance of the wild types used to breed with GA lines are scrutinised.

Archiving of GA lines as frozen embryos or gametes will be encouraged. This not only helps reduce animal use by minimising the number of GA lines maintained on the shelf, but also facilitates the sharing of GA lines between researchers, providing further opportunities for reduction. Repositorie's will be available for future use to safe guard against the potential loss of a line which would be difficult to replace.

Refinement

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

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

Mice are the most common and least sentient of the mammalian species used in scientific research. It is possible to alter mouse genes and manipulate the mouse genome to produce models for human diseases or conditions. The extensive range of established disease models and the fact that they share many common physiological processes with humans makes them the species of choice for many bio-medical research projects.
Rats are less commonly used, but are the species of choice for studies that require a physically larger animal or those with a greater ability to learn and problem solve. The genetic manipulation of rats is more difficult than mice, however several important GA and

harmful mutant rat lines exist that are of great value to many bio-medical research projects.

The animals used under this licence will be adults. The offspring produced may be used at any stage in the development, depending upon the scientific need.

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

All projects using animals generated under this project undergo independent ethical review, which requires the applicant to justify their choice of species.

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

Animals will be handled using refined methods e.g. tube handling or cupping. Environmental enrichment will be provided in the form of dust free bedding and nesting, tubes, houses and aspen sticks for gnawing. All animals will be monitored for signs of impaired wellbeing by experienced animal technicians.

The use of gene editing technology such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), will increase success rate and minimise repetition. Non-Surgical Embryo Transfer (NSET) will be explored if the success rate is clearly evidenced.

Aseptic surgical techniques will be used in conjunction with post and peri surgical analgesia as advised by the NVS.

The standard transgenic protocols, methods and reagents have been optimised for mouse, and there are acknowledged benefits from their use. Vasectomy is one example. The scrotal approach to the vas deferens is a much-refined technique over the abdominal approach and will be used in this licence.

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

Manipulating the Mouse Embryo: A Laboratory Manual, fourth adition. CSH Laboratory press. https://science.rspca.org.uk/sciencegroup/researchanimals
https://www.nc3rs.org.uk/3rs-resources/breeding-and-colony-management
https://www.nc3rs.org.uk/3rs-resources/breeding-and-colony-management/sharing-and-archiving-ga- mice

https://www.nc3rs.org.uk/3rs-resources/minimising-use-ga-mice
https://www.nc3rs.org.uk/3rs-resources/breeding-and-colony-management/efficient-management- genetically-altered-mouse
https://www.nc3rs.org.uk/3rs-resources/breeding-and-colony-management/colony-management-best- practice

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

I will continue to meet regularly with our NC3Rs representative via the Animal Welfare and Ethical Review Body (AWERB). Any advances will be discussed, implemented and reviewed with committee members before rolling out to the wider research community. Regular review of guidelines and recommendations e.g. NC3Rs website will be completed. I will attend organised NC3Rs and Institute of Animal Technology (IAT) events.

3. Eye & ear defects, development and repair

Project duration

5 years 0 months

Project purpose

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

Key words

eye development and repair, stem cell and gene therapy, retinal regeneration, deafblindness, ear development and repair

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

This project aims to increase knowledge of the genetics of eye and ear development, relevant to the causes of congenital eye defects and blindness and deafblindness, and to investigate ways of repairing diseased eye tissue and preventing loss of sensory cells in the retina and cochlea.

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

Why is it important to undertake this work?

Eye development begins in the fourth week of life in the human embryo and is completed after birth. It is a complex process that involves the coordinated maturation of several tissues. Disruption of development, or loss of proteins needed for sight, cause malformations and disease. In the UK about 10 in every 10,000 children are blind and in many cases the condition is incurable and the cause of the disease is unknown. Malformations, together with retinal disease, account for over half of the causes of blindness in children in the UK and are a significant cause worldwide. In some conditions blindness is associated with deafness as similar genetic pathways are important during eye and ear development. Identification of the genetic changes underlying these conditions will provide better information for affected families and enables research to discover the biology of the condition and how it differs from normal. Knowledge of the genetic regulation of eye and ear development presents new avenues for treatment and diagnosis. This may include repairing tissue by transplanting new cells into the eye or by promoting diseased tissue to repair itself or by developing therapies to prevent loss of sensory cells.

In most of our investigations genetically modified mice that model human eye and eye/ear diseases and/or that enable the study of particular genes and cells, will be bred, humanely killed and then used as a source of tissues and cells for analysis in the laboratory. We will use in vitro studies whenever possible, but these can only replace parts of whole animal investigations.

When we do investigations using live animals, for example to transplant cells into the eye to repair diseased retina, the results will be relevant to clinical treatments for human disease, because of the similarities of the two systems. In such experiments, cells or pharmacological reagents or vectors will be injected into the eyes or ears of animals under general anaesthetic. Several weeks after surgery, animals are humanely killed and the tissues removed for analysis. These studies will investigate new approaches to treat incurable causes of blindness and deafblindness.

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

This project will contribute to knowledge of the genetic basis of eye disease and deafblindness and to the development of stem cell-based and genetic therapies for the treatment of blindness and deafblindness.

The outputs will be published in open access scientific journals and by communication with patient groups. Primary datasets will be deposited in open access databases as appropriate.

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

Scientists working in the field and patient groups will benefit from access to the increased knowledge generated in the project.

For example, we work closely with patient and family groups to communicate the research progress towards developing treatments for genetic blindness and deafness.

Developing gene therapy for rare diseases, or developing cell or pharmacological therapies for blindness will have large societal and economic impact, improving the quality of life for patients and potentially reducing lifetime healthcare costs.

The approaches used in this project to develop gene therapy for one type of deafblindness may be useful to advance similar technology in other similar conditions.

The approaches being researched in this project have potential for wide application to treat a large number of people with sight loss.

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

I will seek to maximise the outputs of this work by dissemination of new knowledge and careful reporting of findings in open access sources and seeking further funding to develop and translate this work to clinical therapies.

Species and numbers of animals expected to be used

- Pigs: 24
- Minipigs: 24
- Mice: 4,000
- Rats: 96

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

Study of mouse models of human eye and ear disease is vital to advance understanding of the causes of human malformations and diseases causing blindness and deafblindness and for the development of new therapies. The value of mouse models extend to disease affecting sight and hearing. The benefit of mouse studies is the relatively straightforward extrapolation of results to humans, and therefore to clinical disease. Animal welfare will be upheld throughout and the scientifically justified most animal friendly anaesthesia and least invasive administration route will be selected. Mouse models will be studied at all life stages.

The mouse eye and ear are very similar to the human eye and ear respectively. They develop in the same way and have similar structure, genetics and physiology. The development and structure of the retina and cochlea are very similar between humans and other mammals. Mice carrying mutations in different genes develop eye defects and diseases that causes blindness and deafblindness like those caused by equivalent human genes. By studying mice with eye defects or with diseases that causes blindness and deafblindness it is possible to gain insight into the disease process and to develop new approaches for treatment of blindness. It is impossible to directly study the human disease process because of the inaccessibility of human tissue for study.
This work will use mainly mice (embryos, neonates, pregnant females, juveniles and adults) as a model system for research as the mouse eye and ear resemble those of humans and their genomes are very similar.

Larger animal models (rats, pigs, minipigs; juvenile and adult stage) will be used for studies where the small size of the mouse eye precludes its usefulness, for example, for investigation of cell implant therapies suitable for the human eye. The size of a pig eyeball (21.64–23.9 mm) or mini-pig eyeball (20.33 ± 0.88 mm) is comparable to that of a human eyeball (23–24 mm). The rat eye ball (6–7 mm) , is larger than that of a mouse (approximately 3 mm).

For each study the most appropriate larger animal model will be selected, e.g pigs or mini pigs will be used to test retinal implants suitable for implantation in the human eye. Mini pigs may be imported and used for product testing due to their small size, specific gene profiles and specific health status. The eyes of mini pigs or young standard pigs and are similar in size to a childs' eye.

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

The majority of animals will be used for breeding and to provide tissue samples for post mortem analysis.

Most animals will not be used for live experimentation but will be humanely killed. No adverse effects are expected from breeding mice with genetic eye or ear disease. The expected severity is mild for the majority of the animals.

Experimental animals will receive injections of reagents to test therapies for blindness and deafblindness. The project is designed to use minimal numbers of animals.

In most of our investigations genetically modified mice that model human eye and eye/ear diseases and/or that enable the study of particular genes and cells, will be bred and then used as a source of tissues and cells for analysis in the laboratory. We will use in vitro studies whenever possible, but these can only replace parts of whole animal investigations. Most animals will not be used for live experimentation but will be humanely killed and used to provide tissue samples for analysis. When we do investigations using live mice, for example to transplant cells into the eye to repair diseased retina, or to test gene therapies, the results will be relevant to clinical treatments for human disease, because of the similarities of the two systems. In such experiments, cells or pharmacological reagents will be injected into the eyes or ears of animals under general anaesthetic, or animals will be exposed to pharmocological reagents by systemic delivery routes or exposed to raised oxygen levels to model disease or study the effect of particular genes.

The maximum dosing frequency of injections into the mouse/rat eye is 3 injections over 3 weeks (minimum interval 1 day. See also APPENDIX 2).
The maximum dosing frequency of injection into the ear is 1 injection.
The maximum dosing frequency by systemic delivery (oral, sub-cutaneous, intra-muscular, intra- venous, intra peritoneal) is daily for 30 days.
Mice/rats will be restrained by general anaesthesia no more than twice a week and will receive general anaethesia no more than six times during their lifetime.
Several weeks after surgery, animals are humanely killed and the tissues removed for analysis. These studies will investigate new approaches to treat incurable causes of blindness and deafblindness.

Pigs or minipigs will be used only for investigation of cell/biomaterial implant therapies suitable for the human eye. For example, to transplant cells on a scaffold into the eye to repair diseased retina. The results and delivery will be relevant to clinical treatments for human disease, because of the similarities of the two systems and specifically the equivalent size.
In such experiments, cells or pharmacological reagents will be injected into one eye of animals under general anaesthesia or by systemic delivery routes
Pigs will be restrained by general anaesthesia no more than once a fortnight with recovery in between and will receive general anaesthesia no more than six times during their lifetime.

Several weeks after surgery, animals are humanely killed and the tissues removed for analysis. These studies will investigate new approaches to treat incurable causes of blindness.

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

No adverse effects are expected from breeding mice/rats with genetic eye or ear disease. Rodents with eye and ear defects feed and breed normally as they naturally rely heavily on other senses, such as smell and touch e.g. using whiskers.
Typically, animals will experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal). Animals undergoing repeated injections will experience mild transient discomfort which as it occurs repeatedly may cause mild to moderate distress but no lasting harm.

Animals undergoing ear or eye injections under anaesthesia may experience some discomfort after surgery and some mild to moderate pain which will be treated with analgesics. They are expected to recover quickly and will be given painkillers and post-operative care just like people recovering in hospital.

Where administration of substances is required for prolonged periods, animals may have minor surgery to implant a device (such as a mini pump) under the skin that can release a medicine slowly. These animals will experience some discomfort after surgery and some mild to moderate pain which will be treated with analgesics.

Animals may undergo changes in diet (e.g. to make medication more palatable), which are not expected to cause distress. Rodents will be weighed regularly and placed onto normal diet should they lose 15% of their body weight.

Animals will experience mild and transient discomfort from blood sampling.
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.

For large animal recovery models (porcine)

Those protocols involving recovery surgical procedures under anaesthesia are classified as moderate. An expected recovery plan with appropriate monitoring and analgesia will be

clearly defined in each protocol. Pain would be expected to improve throughout the expected recovery period.

With protocols and procedures that involve animals having recovery anaesthesia, some adverse effects for the animals are expected. All animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Any animal not recovered as expected from the surgical procedure within 24 hours (eating, drinking and return to expected behaviour) will be humanely killed. Animals may experience discomfort and pain following surgery. We will monitor this and provide the appropriate level of pain relief and post-operative care. Other effects include weight loss and changes to behaviour and general overall condition. We anticipate these adverse effects being transient and with good monitoring, welfare management, and nutritional supplements these effects can be alleviated.

With our specialist large animal vets, we have established clearly defined humane endpoints in all our models that minimize discomfort and pain to the animals yet allow us to address our scientific questions.

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

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

The expected severity is :
Sub-threshold for the majority of the animals that are genetically altered;
Mild for those genetically altered animals exhibiting a phenotype and injected by standard techniques;

Moderate for those animals receiving eye or ear injections under general anaesthesia to test therapies for blindness and deafblindness.
Mice: 25% moderate, 25% mild, 50% sub-threshold
Rats and pigs and minipigs: 100% moderate (injection under anaesthesia).

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

- Killed

Replacement

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

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

The mouse/rat eye and ear are similar to the human eye and ear. They develop in the same way and have similar structure, genetics and physiology. Mice/rats carrying mutations in different genes develop eye defects and diseases that are like those caused by equivalent human genes. By studying animals with defects it is possible to gain insight into the disease process and to develop new approaches for treatment of blindness or

deafblindness in people. It is impossible to directly study the human disease process because of the inaccessibility of human pathological tissue for study.

Pigs or minipigs will be used only where the small size of the rodent eye precludes investigation of cell/biomaterial implant therapies for blindness suitable for the human eye.

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

We use in vitro human cell models such as organoids or explanted tissue wherever possible.

It is impossible to directly study the effect of therapies on sight and hearing without studying the whole animal.

It is impossible to analyse sight or hearing in current in vitro model systems. In vivo models are also essential to study the immune system and effects on organ physiology.

Why were they not suitable?

Human tissue models are valuable but cannot mimic the response of living tissue to a new therapy and tests of sight and hearing cannot be performed with the use of experimental 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 estimated number of animals is based on detailed experimental design work plans. We have made calculations using typical variations from our own earlier experimentation to calculate minimum numbers of animals to be used whilst ensuring that the results are statistically significant.

Sample sizes for our experiments are estimated from past experiments. Calculations typically show that we need group sizes of 8 to achieve the quality of results we need. 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?

Animal numbers were estimated using the NC3Rs Experimental Design Assistant.
We will employ 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?

A number of approaches are used to minimise animal numbers. These are as follows: Efficient breeding and colony management. Performing small pilot studies with full analysis to inform design of larger experiments. Using both eyes of animals where appppropriate. Optimising tissue collection for multiple assays.
At the end of the experiment, we will harvest as many tissues as possible at post-mortem. If we don't need to analyse the tissues immediately, we will freeze them. Both male and female mice will be used where possible in the experimental design.

Refinement

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

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

Genetically altered animals provide a model of a human disease or disease process. In this project the animals may have impaired sight and/or hearing loss. The animals typically feed and breed normally and do not show signs of pain or distress.

To induce gene expression in animals or to deplete specific cells, some animals will be given substances by mouth, injection, or through food. This will let us to study processes that happen within short time periods of a few hours. Injections will cause a degree of mild/transient pain.

To test treatments to prevent blindness or deafness some animals will have surgery under anaesthetic to deliver therapeutic reagents or devices directly to the eye and inner ear. They are expected to recover quickly and will be given painkillers and post-operative care just like people recovering in hospital.

These procedures will not cause the animals used to suffer lasting pain, suffering, distress, or lasting harm.

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

Treatments will be tested on juvenile and adult animals at stages equivalent to treating children and adults.

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

We use regular monitoring, post-operative care and pain management to minimise the welfare costs (harms) for the animals.

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

There are published guidelines to assist with planning animal research and testing, such as the PREPARE guidelines:
http://journals.sagepub.com/doi/full/10.1177/0023677217724823

Further information about the PREPARE guidelines can be found here:
https://norecopa.no/prepare

Other resources are available 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 regularly check information on NC3Rs website and attend Regional 3Rs symposia and consult with the regional NC3Rs programme manager.

4. Genes underlying neurodegenerative disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Spinal Muscular Atrophy, Neurodegeneration, Vasculature, Genetics

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To understand the pathology of the human neurodegenerative disease, spinal muscular atrophy.

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

Why is it important to undertake this work?

Spinal Muscular Atrophy is a common (1:6000-1:10000 live births) and severe degenerative disease. The progression of the disease varies depending on genetic factors, but most patients will exhibit loss of motor and respiratory function from a few months of age, leading to death in the majority of cases. It is caused by a deficiency of a protein called survival motor neuron (SMN), which is essential for the function of motor neurons in the spinal cord. SMA is one of the leading genetic causes of infant mortality, with the most severe forms of the disease resulting in death within the first few years of life.
The muscle weakness caused by SMA can lead to difficulties with breathing, eating, and movement. SMA can also cause spinal deformities, such as scoliosis, which can further impair mobility and breathing. Research into SMA is crucial to developing new treatments and therapies that can improve the quality of life for those with the disease. Understanding

the pathology of SMA is important in developing strategies for screening, prevention, and genetic counselling.

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

Spinal Muscular Atrophy is regarded and treated as a degeneration of nerves, which it is, but it is not known why the nerves die. We believe the true problem is a failure of the developing blood supply in the central nervous system (and elsewhere in the body) that leads to nerve death. It would also explain the other problems associated with SMA survivors, e.g. necrosis of fingertips and retinal degeneration. At the end of this project, we will have directly tested whether this is the case, leading to fundamental new knowledge and treatment options.

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

Scientists studying SMA will benefit from fundamental new knowledge about the pathology of the disease.
Clinicians studying or treating SMA will benefit from new target tissues for treatment and better understanding of the long term implications of the disease.
There are expensive gene therapies that can allow some patients to survive SMA, but survivors are far from normal with long term symptoms, associated with vascular defects. Patients will benefit from scientific understanding of the importance of vascular defects and, in time, from therapeutic drugs targeted at the degenerating vasculature.

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

The results of this work will be published in international journals and data made publicly available. Data will be presented at scientific and clinical meetings.

Should analysis of the data suggest a fundamental change in our understanding of SMA, press releases will accompany important publications and presentations to ensure maximum dissemination to stakeholders.

SMA is a relatively common genetic disease and there are multiple patient awareness and support groups and charities, including Spinal Muscular Atrophy UK, SMA Europe (who fund this work) and others (some listed at https://spinalmuscularatrophy.net/support-groups). Through meeting with these groups it will be possible to disseminate work widely to clinicians, patients and their families, and others.

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.

Spinal Muscular Atrophy is a severe disease with postnatal onset and high infant mortality. There are a number of mouse models of the condition and we have previously used the 'Taiwanese model' to demonstrate the occurrence of hypoxia in SMA consistent with

patient data. Typically an SMA mutant mice becomes physically symptomatic from 5 days postnatally and without intervention will die before 20 days of age. The mice that we intend to produce are likely to be less sick than this, but in order to understand the early pathology of the disease, we need to work on young postnatal mice.

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

Typically, genetically modified mice with mild or sub-threshold phenotypes will be maintained and mated together according to the breeding regime outlined. Because the disease is autosomal recessive, with no haploinsufficiency, parents of affected mice will suffer no adverse affects. Most mice will typically only used for breeding sufficient to maintain the stocks. 'Vascular SMA' mice will normally be killed by a schedule 1 method without further procedure. Some mice may be administered cell labelling reagents with doses within guidelines agreed with NVS/NACWO advice, typically following published guidance issued by the Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Groupon doi.org/10.1258/0023677011911, prior to being killed for tissue analysis.

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

The large majority of mice will experience no adverse effects.
Mice with vascular SMA have not previously been generated so we cannot fully predict the phenotype. Symptoms of neurodegeneration could occur in the first week after birth and progress until an endpoint, agreed with NVS/NACWO which will not exceed moderate severity. They may be completely normal. The most likely scenario is that they show a level of adverse effect somewhere between no effect and full-SMA. This would reasonably be expected to mimic mild cases of human SMA, with good life expectancy but a moderate reduction in vasculature to the extremities, possibly leading to risk of tail, ear or toe necrosis.

Full SMA mice exhibit reduces growth from about day 5, exhibit progressive loss of motor function and, without intervention, die before day 20 with respiratory failure. Our previous work on full-SMA mice has used pre- and early-symptomatic mice, showing no, mild, or at worse a moderate phenotype. This is the approach that will be taken in this project (described under adverse affects in protocols). It is assumed that vSMA mice will be less badly affected than full-SMA, but there will be regular monitoring of mice for weight loss, behavioural or vascular problems and SK1 at pre- or (if they happen) early symptomatic stages. No mice will be allowed to progress to acute, severe or lethal phenotypes.

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

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

90% of mice will be genetically altered with no phenotype (sub-threshold).

An estimated 10% of mice, with vascular SMA or carrying human SMN2 gene, will in the worst case scenario exhibit a degenerative phenotype which, with appropriate early intervention, is likely to be moderate severity. This is estimated because Mendelian ratios from final crosses will be 25% experimental 'vascular SMA' mice, but will form less than

half the crosses being performed, since the parent strains (which have no phenotype) have to be maintained separately.

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?

SMA is a complex, multi-system disease that cannot be mimicked in vitro or in silico. Our hypothesis is that failure of the vasculature is responsible for the major neurodegenerative aspects of the disease, and the complexity of the interaction between the CNS and vasculature demands that animal models are used.

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

Searches for 'non-animal model of SMA' and 'in vitro model of SMA' and related terms have confirmed that there are no non-animal replacements for the scientific question we want to address. Organotypic mouse spinal cord cultures exist still require animal input and the production of human spinal cord organoids is in its infancy (see e.g. Lee et al., 2022 - https://www.nature.com/articles/s41551-022- 00868-4). Searches for vascularised spinal cord organoids have produced no hits. Searches for Spinal muscular atrophy vascular culture produces only animal model research (the major paper by our co-grantholder). Bioinformatic tools exist to identify alleles of SMN2 that may modulate disease function or predict drugable targets, but they do not address disease etiology (doi: https://doi.org/10.1038/d41586- 023-00906-4). A summary of current in vitro tools for SMA research is published here https://smafoundation.org/discovery/in-vitro-tools/.

Why were they not suitable?

It may be possible in the medium future to develop an organoid with normal CNS tissue and SMA- mutant vasculature, but this will never recapitulate the complexity of the disease-causing process and cannot account for spatial variability of the vasculature and neurones in different parts of the spinal cord. We have performed in vitro work and already know that SMA mutation affects vascular cells in tissue culture, and there is no further we can go without animal work. Even if non-animal spinal cord organoids could be produced the techniques required to vascularise them currently do not exist, though some progress has been made (see for example Shi et al., 202W0 - https://journals.plos.org/plosbiology/article?id=10.1371%2Fjournal.pbio.3000705). We share labspace and collaborate with a neighbouring research group who are developing vascularised human CNS organoids, and we have the opportuny to help develop an SMA in vitro model in future. However, even if we or someone were to develop a human spinal cord organoid with functional motor neurones and a functional essential vasculature that we could incorporate SMA mutations into, this one only address one facet of the complex multi-system disorder that we are trying to replicate in animals. Similarly non of the listed in vitro tools at https://smafoundation.org/discovery/in-vitro-tools/ address this research question.

Reduction

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

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

We estimate using 1000 animals in the course of developing the breeding stock and initial analyses of disease phenotypes described in this project. We need to import a breeding nucleus (2 male 2 female) of 4 stocks of mice (1) SMNFl/Fl 2) Tie-2Cre, 3) SMN-/- SMN2Hu/Hu 4) SMN+/-). We will breed stocks 1) and 3) together to produce a nucleus of SMNFl/- SMN2Hu/- mice (Stock 5, 32 mice). We will inbreed stock 5) to produce male and female SMNFl/Fl SMN2Hu/Hu mice (stock 6). These will only be 1/16 of the litters so we need 10 or 12 litters (80-120 mice) to get at least 2 male and 2 female. These mice will then be maintained by inbreeding and Stocks 1, 3, 4, 5 will be discarded.
Separately we will breed Stocks 2 and 3 to create stock Tie2-CreTg/- SMN+/- mice (stock 7) (~64 mice) that will be bred with Stock 6 routinely during the project to create litters when 1 in 4 mice are the vascular SMA vSMA model Tie2-CreTg/- SMN-/Fl SMN2Hu/- (~400 mice = 100 vSMA for analysis).

Stocks 2 and 3 will have to be maintained as basic minimal stocks for the duration of the project (~400 mice) in order to provide healthy parents for the experimental mouse crosses.

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

We have optimised the breeding regime of mice to minimise the number of mice of incorrect genotypes that are bred, and to maximise the number of vSMA mice produced with minimum number of matings.

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

Rapid and accurate genotyping using published techniques will ensure that mouse stocks will be managed most efficiently. There will be some uncertainty in relation to numbers of mice used due to stochastic variation in Mendelian ratios produced during crosses, but this will be managed and monitored weekly. Tissues from excess controls or wrong genotypes will be used for other projects where possible.

Refinement

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

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

We will genetically modified mice that have a conditional mutation of the SMN genes only in the vasculature. Other tissues of the mice have normal SMN activity.
Total loss of SMN in these models in all tissues (which is the model we have previously used) causes death within 3 weeks of birth. The vascular-only defect will certainly not be more severe than this, and in all likelihood will be considerably less severe.

The breeding regime uses pre-existing strains of mice and will not require de novo creation of new mutations, thus avoiding the extra animal work associated with genome editing.

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

There is a zebrafish model of SMA which mimics the mouse models (Hao et al., 2011 - https://molecularneurodegeneration.biomedcentral.com/articles/10.1186/1750-1326-6-24). This model of zebrafish SMA has all of the components of human SMA and can thus be used to understand motoneuron dysfunction in SMA, can be used as an vivo test for drugs or antisense approaches that increase full-length SMN, and can be developed for drug screening. It does not however, have a floxed Smn gene that could be used to make conditional knockouts - i.e. it is a 'full SMA model that affects all tissue, not just vasculature. Although it is conceivable that a vascular Cre fish coould be made driven from a Fli1 gene promoter (Delov et al., 2014 - https://pubmed.ncbi.nlm.nih.gov/24685623/), floxing of zebrafish genes, which would be required for tissue-specific knockoit of smn, is not yet a mainstream technology. A vSMA fish does not therefore exist and would need a significant advance in technology to make it so. There are therefore no non-mouse models of SMA in which to investigate nervous system-vasculature interaction, so less sentient models are not an option. We need to follow the progress of the postnatal phenotype which requires keeping the mice alive. Where possible, genetically modified animals that may be carrying disease alleles will be used in experiments before birth to avoid possible suffering.

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

Daily monitoring of animals who may be carrying deleterious mutation in the SMN1 gene will be performed, with endpoints agreed in consultation with animal facility and veterinary staff. Scoring sheets will be used to monitor health and weight of mice during procedures such that any health concerns are quickly highlighted and dealt with. Appropriate housing modifications including soft bedding and if necessary, soft food would be used where required.

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

The proposed research has been designed with consideration of the 'Responsibility in the Use of Animals in Bioscience Research' and the guidleines and tools published by National Centre for the Replacement, Refinement and Reduction of Animals in Research 3Rs (replacement, reduction and refinement). These include experimental design assistant https://eda.nc3rs.org.uk/ and the resources on husbandry, refinement, in vivo techniques and genetic modification available at https://www.nc3rs.org.uk/3rs-resources

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

PPL applicant is a member of the NC3Rs project grant funding panel and receives regular updates through this channel about 3Rs advances and latest development and thinking in 3Rs. Any new information that can be implemented in terms of more refined genetic models, welfare practices or technical experimental procedures will be incorporated, where possible, into the programme of work in consultation with the Named People.

5. Glucocorticoids and Cardiovascular Disease Risk

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

Obesity, Insulin Resistance, Diabetes, Hypertension, Glucocorticoids

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To identify the mechanisms that regulate systemic and local glucocorticoid action, and determine their contribution to the pathophysiology of cardiovascular disease risk .

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

Why is it important to undertake this work?

Cardiovascular disease remains the leading cause of mortality worldwide. The increased prevalence of obesity and it's associated co-morbidities including insulin resistance and hypertension are drivers of cardiovascular disease. Therefore understanding the mechanisms that underly the development of these conditions is crucial in order to identify therapeutic opportunities to prevent cardiovascular disease-related deaths.

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

This research will deliver new knowledge of the pathways that control glucocorticoid action in tissues important to cardiovascular disease risk, including adipose tissue, liver, skeletal muscle, vessels, and brain. This knowledge will be disseminated widely through presentations, publication in peer-reviewed open-access journals, and public engagement forums.

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

Scientific Community: The primary beneficiaries of this in vivo project are the scientific and research communities. The project aims to identify and elucidate the mechanisms that regulate systemic and local glucocorticoid action in the context of cardiovascular disease risk. The knowledge generated from this research will contribute to a deeper understanding of the pathophysiology of cardiovascular disease and its relationship with glucocorticoids. Researchers and scientists in the field of cardiovascular physiology, endocrinology, and related disciplines will benefit by gaining new insights into these mechanisms.

Medical and Healthcare Professionals: Healthcare professionals, including physicians and clinicians, stand to benefit from the project's outputs. Understanding the mechanisms linking glucocorticoids, obesity, insulin resistance, and hypertension to cardiovascular disease risk can inform clinical practice. It may lead to improved risk assessment, diagnosis, and treatment strategies for cardiovascular diseases, ultimately benefiting patients by enhancing the quality of healthcare and potentially reducing mortality rates. Patients: Patients with or at risk of cardiovascular diseases are indirect beneficiaries. The project's findings could lead to the development of new therapeutic approaches and interventions. This may result in better management of cardiovascular risk factors, such as obesity, insulin resistance, and hypertension, and potentially reduce the incidence of cardiovascular disease-related deaths. Patients may experience improved health outcomes and a higher quality of life as a result.

Public Health Authorities: Public health authorities and policymakers may benefit from the project's outputs in several ways. Understanding the mechanisms involved in cardiovascular disease risk can inform public health policies and interventions aimed at reducing the prevalence of risk factors like obesity. It may lead to targeted prevention strategies, health education campaigns, and policies that promote cardiovascular health. This could ultimately reduce the societal burden of cardiovascular diseases and associated healthcare costs.

Pharmaceutical Industry: The pharmaceutical industry may benefit from the identification of new therapeutic targets and mechanisms related to glucocorticoid action in cardiovascular disease. If novel drug targets are discovered, pharmaceutical companies could potentially develop and market new medications or therapies to address cardiovascular disease risk factors more effectively.

In summary, the outputs of this in vivo project have the potential to benefit a wide range of stakeholders, including the scientific community, healthcare professionals, patients, public health authorities, and the pharmaceutical industry. The knowledge gained from the research can lead to better understanding, prevention, and management of cardiovascular diseases, ultimately improving public health and patient outcomes.

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

The work generated from this research will be presented at national and international scientific conferences and published in peer-reviewed open-access journals. Datasets generated will be made publicly available to other researchers. We will seek to build upon our current collaborations to establish new connections with local and international researchers, with the aim of ensuring mutually beneficial outcomes.

Species and numbers of animals expected to be used

- Mice: 4000

Predicted harms

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

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

Mice are selected as the experimental model because this species has been extensively used as a model organism in the understanding of human metabolic diseases. Importantly, gene targeting technology and dietary manipulation is widely available for the mouse and thus allows investigators to precisely establish casual relationships between genes and biological processes. Phenotyping must be carried out on an adult animal because not all features of the mature mammalian cardiovascular system can be replicated via in vitro methods.

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

While a number of procedures are listed in this project, animals will not undergo all of the procedures.

Animals will undergo changes in diet which are not expected to cause distress but may sometimes result in obesity. Some diets may result in weight loss due to unpalatability. Animals will be placed onto normal diet should they lose 20% of their body weight. Typically, animals will experience mild, transient pain and no lasting harm from administration of substances by injection. Where administration is required for prolonged periods, animals will be surgically implanted with slow release devices such as a mini-pump. These surgeries may coincide with removal of adrenal glands which produce steroid hormones such as glucocorticoids, or the removal of ovaries or testes which produce sex hormones. In some instances, a separate or additional surgery may be required in other to implant vascular catheters and allow vascular access. These animals can experience some discomfort after surgery and some mild to moderate pain but peri- and post-operative pain-relief will be provided. In the case of adrenal removal, an inability to control blood pressure may occur due to lack of mineralocorticoid hormones. This is prevented by provision of saline in drinking water. Animals will experience mild and transient discomfort from blood sampling.

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

Animals will have varying levels of surgery ranging from implanting a device under skin to release drugs to mode removal of non-essential tissues (adrenals, ovaries, testes) and

catheterisation of arteries. They are expected to recover quickly and will be given analgesia peri- and post-operatively. Animals will be fed varying diets that may result in weight gain (obesity), but are not expected to cause distress.

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

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

Overall:
Sub-threshold: 45% mice

Mild: 20% mice

Moderate: 35% mice

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?

Glucocorticoid hormones affect all aspects of physiology, particularly the cardiovascular and immune systems. They regulate metabolism. Where possible, we use in vitro systems to investigate direct effects of glucocorticoids or of their metabolism/transport in a particular cell type (eg primary cultures of adipocytes, neutrophils, cell lines). However, cell lines or primary cells cannot replicate the systemic effects of cell products (including of glucocorticoid metabolism, for example) upon other tissues, or upon parameters such as blood pressure or insulin resistance. Measurement of insulin sensitivity, blood pressure, glucocorticoid-delivery to target cells cannot be performed ex vivo using existing methodology. This requires an in vivo approach, although much of the analysis is carried out ex vivo. In silico approaches continue to be used, in particular for newly identified glucocorticoid metabolites, to determine their potential interactions with receptors/binding proteins etc. However, animal models are necessary to provide the data for validating and refining computational models of glucocorticoid physiology.

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

Cell lines and primary cells will be utilised to look at cell-specific regulation of glucocorticoid action. Co- cultures of cells will also be utilised when assessing interactions between cell types. In silico approaches will be utilised initially if novel glucocorticoid metabolites are identified from this work.
Zebrafish models have also been considered, however as outlined below are not suitable.

Why were they not suitable?

While cell-based systems can provide improtant pathway information, a key component of this rsearch relies on dissecting the interactions between different tissues (eg how changes in adipose tissue glucocorticoid action impacts liver glucose production). Modelling these complex systemic interactions is not currently possible in in vitro cell-based settings. Similarly, computational modelling of novel glucocorticoid metabolite interactions provides valuable information for informing research strategy, but requires validation in an in vivo setting. Zebrafish have been considered as an alternative approach, however they lack the major glucocorticoid binding protein that is a central component of our research, and are therefore not a suitable 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?

Data from previous work has been used to estimate the number of animals required. At each stage we scrutinise experimental design and present our experimental design within our research group to provide opportunities for others to utilise tissue/data from our experiments to increase their impact.
Control groups are assessed and identified based upon the research question, ensuring that robust, appropriate controls are utilised.

All experiments will have a randomised design where possible, in which mice will be randomised to treatment groups (e.g. pharmacological treatment) using Excel. For downstream analyses, we will be blinded to the genotype, sex and/or treatment group by a lab member, and samples processed using coded identifiers. Data will be uncoded post-analysis.

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

Planned and current experiments are discussed at our regular “data” and at “steroid interest group” meetings. This increases scrutiny of experimental design and presents opportunities for others to utilise tissue/data from experiments to increase impact of experiments.

Experimental design, relevant to all aims/objectives: Our experiments are designed and carried out in accordance with the ARRIVE and the PREPARE guidelines. Study design is based on current best practice, including utilisation of the Experimental Design Assistant of the NC3Rs (https://www.nc3rs.org.uk/experimental-design-assistant-eda). Data from previous work has been interrogated to determine appropriate sample sizes and statistical analyses.

Control of variability: Using inbred mice reduces inter-animal variability and thus overall numbers required. In some cases with GA mice, experimental and control animals are

caged together, reducing variability. Glucocorticoids regulate stress responses, including responses to injection or surgery.

Therefore, in some experiments it is necessary to include a non-manipulated (eg non-operated/non- injected) as well as a sham-operated/vehicle-injected group of animals as the control. In vivo imaging allows sequential non-invasive measurements within a single animal, increasing statistical power and reducing the number of animals required for experiments. Moreover we have developed sensitive techniques that allow parallel comparison of experimental endpoints within the same mice. This includes assessment of insulin sensitivity and assessment of systemic/tissue glucocorticoid levels.

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

Breeding: Breeding of animals is a major part of this project. Mice are genotyped as soon as possible after weaning and only useful genotypes are maintained. In some cases, wild-type mice from breeding Protocol 1 (following genotyping) will undergo continued use in one of the other protocols. Male transgenic mice (which retain fertility longer than females) are used to maintain a line and ensure the minimum number of animals are used in breeding programmes.

Pilot studies: Where there is no or little information (eg. the dose required for a molecule/drug intervention), pilot studies will be performed according to best practice (https://www.nc3rs.org.uk/conducting-pilot-study) using a few animals to determine whether the level of intervention is sufficient, to check for unexpected toxicity or adverse effects of a treatment or procedure, to check for the effectiveness of actions to reduce any adverse effects and to define early humane end-points. Dosing regimes will be determined using information obtained from effective concentrations in vitro, and from the literature where available.

Experimental design is discussed within our local research groups prior to any study, maximising the potential for other researchers to acquire tissue/data. At the end of experiments, as many tissues as possible are harvested and stored appropriately to ensure their availability to our group and 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.

To study the effect of glucocorticoids on cardiovascular disease risk, we utilise a number of genetic, surgical, and pharmacological approaches.

Genetically altered mice, especially those with tissue- or temporal-specific alterations, allow powerful investigation of mechanism by intervention that is difficult or impossible to achieve by other means. Where the genetic alteration is the sole intervention (with analysis ex vivo), overall animal suffering may be reduced, compared with other types of intervention. Breeding strategies in which the male is transgenic and the female non-transgenic are normally used to maintain as near normal a maternal environment as possible.

To study the impact of obesity, dietary manipulation will be required. The impact of diet in these models will be assessed over the minimum time period possible. Techniques to study the outcome of dietary interventions focus on the use of 'gold-standard' measures that yield multiple data sets, maximising outputs. Some animals will be given substances by mouth or injection. In some cases substance administration will be via implantation, and which will require surgery. In any animal requiring surgery, strict guidelines pertaining to post-operative care and monitoring will adhered to in order to minimise potential suffering.

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

To accurately study the physiology and pathophysiology of glucocorticoid action in the setting of cardiovascular disease risk, our research requires the use of mature, post-weaning age animals to ensure accurate recapitulation of the human setting. Species such as zebrafish have been explored for their potential, however they are a poor model of human physiology as they lack a major protein involved in glucocorticoid physiology. Terminal anaesthesia interferes with the stress response, inducing changes in glucocorticoids that would hamper interpretation of results.

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

Where possible, protocols are refined. By extracting steroids from urine-soaked absorbent material placed in cages, we avoid using metabolic cages to collect urine to measure ratios of steroid metabolites. We have refined the adrenalectomy protocol to include steroid replacement from the time of adrenalectomy as far as experimental design allows, minimising the possibility of an adrenal crisis which can occur due to lack of glucocorticoids. We have refined ways of blood sampling from the tail, avoiding the use of restraint tubes and performing it more quickly to minimise stress to the animal. We have refined our measurement of blood pressure by training mice for a number of days before measurement to minimise stress and reduce variability in our data.

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

Our experiments are designed and carried out in accordance with the ARRIVE and the PREPARE guidelines. Study design is based on current best practice, including utilisation of the Experimental Design Assistant of the NC3Rs.

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 in the process of our experimental design, liaise with veterinary staff to ensure information is up-to-date. An

Experimental Request Form is submitted prior to starting each experiment. This prompts discussion with the NVS of additional refinements that could be applied within the protocol. Where experimental design allows, these are always incorporated.

6. Information processing in the mammalian brain

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Neuron, Brain, Behaviour, Imaging, Electrophysiology

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To understand mechanisms of information processing and neural plasticity at the single-cell level and in populations of interconnected neurons and to probe how these mechanisms contribute to learning and behaviour.

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

Why is it important to undertake this work?

The results of this project will extend our basic knowledge of the fundamental mechanisms underlying information processing and memory storage in mammalian central neurons. This is essential if we are to understand how neurons communicate with each other and how information is transformed and stored by networks of neurons in the intact brain and will provide a framework for probing the mechanisms that cause dysfunctional brain circuits in disease.

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

This project will provide a wide range of new information about how cellular and network properties in neurons of different brain areas are involved in learning and behaviour. This includes:

1. An unprecedented dataset of behavioural and physiology measurements

2. Technical advances in approaches to probing the activity of populations of neurons during behaviour, including high density electrophysiology recordings, and manipulation of activity using optical methods

3. Peer reviewed scientific publications about new findings and new methods

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

In the short term, new data and technological advances will benefit understanding and enable advances in experimental approaches, including refinements to the use of animal models, such as methods to increase data gathered for each animal used and modelling to make predictions that refine experimental design and reduce the total number of animals used. Data will be shared with collaborators and uploaded to data sharing platforms to benefit other researchers.

In the long term the results of these experiments, and the techniques we will have developed, will provide new approaches of potential value for understanding and treating disorders of brain function such as occur in stroke, hereditary movement disorders, epilepsy and dementia

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

1. In addition to publication in open access peer reviewed journals, work will be made available as preprints on BioRxiv and will be communicated via posters and talks at scientific meetings and other institutions worldwide.

2. All data will be made freely available to the scientific community through dedicated internet-based repositories

3. All technical advances will be made available by publication and deposition of materials and methods via appropriate open access platforms

Species and numbers of animals expected to be used

- Mice: 19,000
- Rats: 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.

The experiments in this licence aim to improve our understanding of the mechanisms of neural information processing and learning in the mammalian brain during behaviour.

Rodents are probably the lowest species for which direct comparison can be made with the structure and functioning of the human brain. Mice are currently the species of choice in most areas of biomedical research, as they allow the use of powerful techniques such as the generation of transgenic animals. By using transgenic animals, we can express non-harmful molecules, such as fluorescent proteins, in neuron types of interest, that allow us to look at and manipulate, in a very targeted way, the activity of cell types most relevant to our research questions, rather than just sampling all cells randomly. This greatly enhances the scientific value of the work as well as reducing the number of animals needed for experiments. As more sophisticated genetic targeting methods are introduced, we will use them to further refine our scientific approach in order to gather data even more efficiently. The majority of the project will use young adult (approx postnatal day 60) to adult mice, when the circuitry of brain areas of interest is fully developed. Recently, transgenic rats have also been successfully bred, and the introduction of these into the project will allow experiments to be done that are directly comparable to previous studies using rats and permit the use of imaging devices in freely moving animals that are not yet light enough to use in mice.

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

The project can be divided into two main categories: breeding of transgenic mice and experimental procedures. Typically, breeding will not involve any additional interventions. Experiments will usually start with a surgical procedure under anaesthesia to install a head plate onto the skull of the animal, along with creation of a small window in the skull and an injection of substances (viral vectors to express harmless fluorescent molecules) into the brain. Everything will be secured with dental cement and wounds closed. After recovery of 4 days to 2 weeks animals will be acclimatised to head-fixation and will be trained on a behavioural task, if required. Animals will then carry out the task on a recording apparatus, or be acclimatised to sit passively and receive sensory stimuli, usually under an imaging or electrophysiology system and the activity of brain cells will be recorded and/or manipulated using light. In approximately 50% of cases, animals will be subject to changes in their diet or water restriction in order to motivate learning. The typical length of an experiment is 4-6 months. Experiments in anaesthetised animals and from in vitro brain slices will be used to gather data where possible, for example delivering ground truth data on single cell and circuit effects of optogenetic stimulation.

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

Animals may experience transient post-surgical pain and discomfort which will be monitored and ameliorated by using appropriate analgesia until full recovery and as needed. Initially, when subjected to head fixation, animals experience discomfort/anxiety, so they will be gradually acclimatised to this while being rewarded, until they are comfortable and able to sustain periods of head restraint compatible with experimental conditions. Animals that have their food or water rationed so that they will take either food or water as a reward during the experiment may lose weight of up to 20% of normal age-matched animals, but this will usually stabilise at 15% with no signs of detriment to general health and welfare. Animals will be very closely monitored to ensure that they do not lose more than 15% of their normal body weight and they do not show any signs of ill health. If an animal loses more than 15% of their normal body weight or any adverse effects during dietary restriction are observed, a normal diet and water intake will immediately be reinstated.

Expected severity categories and the 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 Mouse 5% Rat 45%

Mild Mouse 75% Rat 0%

Moderate Mouse 20% Rat 55%

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 main goal of the project is to examine the computations taking place in neural circuits in the functioning mammalian brain during sensory processing, learning and performance of behavioural tasks. To achieve this goal it is essential to perform work on intact animals. We complement in vivo work with in vitro brain slice preparations where a suitable proportion of the circuitry remains intact and where some elements of experiments can be better controlled. We also use advanced fixed tissue methods to examine the structure of circuitry at the electron microscopic level.

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

Whenever possible we use computer models constrained by our experimental data to guide our experimental design, to extend our understanding of our data and to minimise the number of animals used in experimental procedures.

Why were they not suitable?

The mammalian brain is extremely complex, and understanding its functioning requires experimentation at different levels, ranging from molecular and cellular to systems and behaviour. While computer models can simulate some of these levels of complexity, they are limited in their ability to replicate the full range of biological processes that occur in living organisms. Computer models need to be validated against real-world data to ensure their accuracy and reliability. This requires experiments on living organisms, including animals, to gather the necessary data. Computer models can complement animal experiments by helping to integrate data from different sources and levels of complexity. However, it is difficult to develop a computer model that captures the full range of biological processes involved in the functioning of the brain without the input of experimental data obtained from 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?

All mouse numbers are based on the number breeding pairs required to generate sufficient mice for experiments and on the assumption of a 25% success rate in performing all the necessary procedures required, training to adequate levels of task performance and the likelihood of any mouse fulfilling all the practical requirements for each experiment (e.g. expression levels of indicators and opsins). This rate is estimated from 15 years of experience of carrying out such experiments. Mouse experimental pipelines are usually carried out in cohorts, to reduce variability from changes in environmental and other conditions, and multiple breeding pairs are needed to supply the mice required. A small number of mice may be used for pilot studies to refine co-ordinates for stereotaxic injection. Blinding and randomization will be done through automated software pipelines that either hide identifying information about the data during manual data analysis or analysis will be automated so that it can be carried out without human intervention. Because we use a large number of different mouse lines (both cre and reporter lines) and breed these, sometimes creating triple transgenic strains, to allow specific targeting of identified populations, breeding numbers for this project are substantial.

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

For most experiments the same mouse can be used for obtaining experimental and control data, reducing the number of animals needed and reducing variability while increasing statistical sensitivity. Large amounts of data will be gathered multiple times from each mouse, as the techniques used (2- photon imaging, Neuropixels) provide the power to record from several hundreds of neurons simultaneously and to perform repeated experiments over a number of weeks. By carefully mapping activity in each mouse, we can record from the same neurons repeatedly, allowing paired comparisons to be made in different conditions (e.g. during learning, across behavioural tasks, with/without perturbation), further increasing the statistical sensitivity and power of the data collected from each animal. In addition, some mice can be included in multiple project aims and increase the impact of the data. We will seek guidance from NC3R resources to continually evaluate 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?

Breeding will be carefully controlled so that only animals required for experiments are generated. Mouse experimental pipelines are usually carried out in cohorts, to reduce variability from changes in environmental and other conditions, and multiple breeding pairs are needed to supply the mice required. However, this also means that, after mice have been produced for a particular set of experiments, breeding numbers can be reduced. We will share mice if appropriate surplus stock is available. We will use the latest algorithms

for data analysis such as spike sorting (e.g. Kilosort) and clustering algorithms (e.g. Suite2P) to extract the maximal amount of information from each imaging and electrophysiology experiment, reducing the number of animals used.

Refinement

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

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

All our experiments will be done in rodents (mouse and rat), which are the phylogenetically lowest species that have behaviours and brain structures directly comparable to humans. Two key advantages of these rodent models are 1) the potential for using transgenic animals, which allow highly refined experimental designs that target genetically identified populations of neurons; 2) a vast body of literature on mouse and rat behaviour and the underlying neurobiology, which again allow for highly refined experiments. Our methodological approaches, which we have refined over the past 20 years, are designed to ensure that animal behaviour remains as normal as possible and that animals are able to engage with behavioural tasks to learn and perform them effectively. It is a requirement, therefore, that measures are taken to ensure that animals are not suffering from pain or distress at the time of the experiment and that these are minimised, using general constraints, during the preparation process.

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

Our scientific aims need to be addressed at a stage where the animal is fully developed, to avoid variability that may occur as part of the developmental process. We therefore use late juvenile to adult animals. Although sensory responses can be tested under anaesthesia, it is well known that anaesthetic drugs affect activity in neural circuits and may provide misleading results. It is also not possible to use terminally anaesthetised animals to evaluate neural activity during behaviour and changes in neural activity and circuitry during learning. For these reasons, although some of our experiments will be carried out in acute brain slices or in terminally anaesthetised animals, it will remain necessary to work with awake, behaving animals.

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

We are continuously refining our approaches to enable the selective expression of specific molecules, such as fluorescent proteins, in identified populations of neurons to facilitate target-directed recordings. So far we have done this by using mouse lines expressing cre-recombinase in specific cell types and injecting virally-packaged DNA fragment containing the gene in an arrangement that means it will only be expressed if it gets into cells containing Cre. We will take advantage of new strains of transgenic mice that do not require injections of virus to provide expression of reporter proteins or optogenetic molecules but that can instead be provided by breeding or by pharmacological induction.

This will reduce surgery duration and/or number of surgeries required to prepare the animal for experiments.

We continuously refine our recording configurations, including streamlining head restraint systems to minimise the weight and maximise ergonomics so as to minimise any physical impediment to the mouse in the home cage. We will continue optimising and adding to these refinements.

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

PREPARE: guidelines for planning animal research and testing Smith AJ et al Lab Anim 2018 52 (2) 135-141

LASA guidance

NC3Rs recommendations including: Refinements to rodent head fixation and fluid/food control for neuroscience. Barkus C et al., J Neurosci Methods. 2022 Nov 1; 381:109795

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

I will keep up to date with 3R developments through the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs), including recommendations for best practices in neuroscience rodent experiments. I will further be informed by the local 3Rs group at the establishment as well as animal facility staff and Named Persons, who I will work closely with to continuously implement 3Rs advances.

7. Profiling of CNS action of novel drugs by neurochemical sampling

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

Microdialysis, Brain, Chemicals, Drug Development, Neurochemistry

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of this project is to provide highly specialised preclinical data to our clients to support either the development of novel, candidate compounds for the treatment of central nervous system (CNS) and metabolic disorders or to assess compounds from non-CNS-related projects for centrally mediated 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?

CNS disorders encompass a wide range of mental (e.g., depression, anxiety, psychosis, attention/behaviour disorders, autism, Down's syndrome, sleep disorders and drug abuse), neurological (e.g., Alzheimer's disease, Parkinson's disease, Huntington's disease and epilepsy) conditions that negatively impact mood, behaviour, brain functioning, cognition, sensory or motor function and metabolic disorders, including obesity and diabetes. These disorders are highly prevalent worldwide affecting hundreds of millions of people in both developed and developing countries. For example, approximately 280 million people in the world suffer from depression (http://www.who.int/news-room/fact-sheets/detail/depression), 24 million from schizophrenia (http://www.who.int/news-room/fact-sheets/detail/schizophrenia), 50 million from epilepsy (http://www.who.int/news-room/fact-sheets/detail/epilepsy), 55 million from dementia (http://www.who.int/news-room/fact-sheets/detail/dementia), 422 million from diabetes (http://www.who.int/news-room/fact-sheets/detail/diabetes) and 650 million adults from obesity (http://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight). They produce short- or long- term impairments and disabilities and therefore are an emotional and financial burden to patients, their friends and families and society as a whole. Furthermore, there is a strong link between psychiatric conditions, such as depression and schizophrenia, and suicide. Also, those with mental illness are at high risk for developing other chronic conditions such as cardiovascular disease, respiratory disease and diabetes. In response to the low levels of investment in these noncommunicable diseases (NCDs) globally, even though they cause three quarters of deaths worldwide, the World Health Organization has launched the Global NCD Compact 2020-2030 (https://www.who.int/initiatives/global- noncommunicable-diseases-compact-2020-2030). The aim is to accelerate progress on prevention and control of NCDs by ensuring that member states adopt policies and programmes that improve outcomes and save the lives of patients living with NCDs.

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

The benefits from this project will relate to the three types of data that will be generated:

Mode of action/efficacy: neurochemical data (levels of neurotransmitters from distinct brain regions)

Side effect data: neurochemical data and behavioural data

Pharmacokinetics (PK): the fate of a drug within the body can be determined by tissue (e.g., blood, plasma, brain) levels of that compound over time after administration.

The likelihood of achieving these benefits is high. The data generated in this project will be used to provide our clients (principally from the pharmaceutical industry) with data to support their development of candidate compounds with potential for the treatment of CNS and metabolic disorders. The data may be used to decide whether or not a compound can progress to clinical trials and may be included in the data submitted to Regulatory Authorities for licensing new treatments.

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

In the short term our clients whose compounds we test will benefit in terms of providing useful data to determine whether or not a compound can progress to clinical trials. In the long term this work may lead to the approval of new treatments for the type of conditions outlined in the Aims section.

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

Although our work is client confidential, we have a commitment to the dissemination of information into the scientific community and findings will be made available to other scientists through publication in peer-reviewed journals and presentations at scientific conferences and meetings. Such information is not solely in regard to distributing knowledge about the efficacy and safety of particular agents/classes of compound to treat CNS and metabolic disorders but is also in regard to refined surgical methods.

This can be at a local level (e.g., our work involving specialised mouthpieces for mouse anaesthesia helped refine procedures of other groups at the University) or at an international level.

Species and numbers of animals expected to be used

- Mice: 450
- Rats: 2600

Predicted harms

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

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

Rats and mice are the lowest sentient animals that can be used for assessing neurochemical effects of novel compounds in a whole brain system in a manner that is translatable to humans. We will be using adult animals, which are needed for long term intracranial implantation of guide cannulae and/or probes, as we need the skull and brain to be fully formed to prevent any pain from bone growth being restricted by the cemented implant.

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

In the majority of studies, rats and mice will undergo surgery using an anaesthetic, typically lasting 0.5-

1.5 hours. Very small incisions will be made in the skull to gain access to the brain and tiny screws and microdialysis probes will be implanted into specific brain areas. Dental cement is used to secure the implants and seal the skull surface, the skin is sutured and animals are placed in cages to recover. Pain relief is administered and animals are observed closely for any signs of pain or discomfort. Recovery from the anaesthetic is usually quick, e.g., within 30 minutes. The probes will be perfused with a liquid which is collected and analysed for chemicals. Drugs will be administered, usually once, and samples collected before and after. Occasionally, in rats only, a catheter will be implanted into up to 2 vessels (e.g., jugular and/or femoral vein). This can either be at the same time as the surgery to implant the probe or in a second anaesthesia session after a 2-week recovery period from guide cannula or probe implantation. The rats will have blood samples collected from the catheter at various time intervals so that levels of drug in the blood stream at various time points post administration can be measured. Most experiments will

last for 4-6 hours. At the end of procedures rats and mice will be humanely killed to allow post-mortem examination and collection of tissues for analysis, if required.

PK studies will involve administration of the candidate substances and the taking of biological samples (e.g., blood) from live rodents at pre-determined time points and tissue (e.g., brain, cerebrospinal fluid) after humane killing of the animal.

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

All animals undergoing surgery are expected to recover quickly and will be given painkillers and post- operative care just like people recovering in hospital. Their health and body weight will be monitored. The administration of compounds for testing will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort (e.g., small prick from insertion of a needle) and no lasting harm. The compounds may result in CNS-mediated behaviours following their administration (e.g., increased motion around the cage) but this should be transient (e.g., 1 to 2 hours).

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

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

Mild - 5% for both rats and mice

Moderate - 95% for both rats and mice

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

- Killed

Replacement

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

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

The project aims require investigation of candidate compounds to be tested in an integrated behavioural/physiological/pharmacological model that requires the whole animal. Although every effort is made to use in vitro systems e.g., the use of cell lines, cells and tissue cultures, to replace in vivo animal experiments, it is accepted that whilst neurotransmitter release and reuptake experiments in vitro provide valuable information on drug mechanisms, these simplified model systems lack the neuronal complexity and control mechanisms present in the brain. As a consequence, they cannot provide the level of assurance and validity that is obtained by the use of intracerebral neurochemical sampling where overall effects on the intact animal can be observed. Prior to testing in the present project, it is expected that candidate compounds will be selected on the basis of in silico (computer modelling), in vitro and ex vivo (using intact tissues from humans or animals) experiments. Importantly, although these experiments provide clients with

important information in regard to the discovery of candidate compounds, the data relate to discrete parts of the animal and are not suitable for replacing in vivo tests in whole animals, such as those to be carried out in this project.

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

It is not possible to use non-animal alternatives for this project due to the nature of the data acquired as outlined above.

Why were they not suitable?

This is because there are no in silico or in vitro model systems that can adequately model the living brain and body to be able to acquire the type of data we propose to be collecting in this project. Also, regulatory authorities expect a drug's sponsor to have screened new molecules for pharmacological activity and acute toxicity potential in animals, prior to assessing its diagnostic or therapeutic potential in humans. The data produced by this project for our clients helps fulfil that expectation.

Reduction

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

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

In our extensive experience in this field, a typical microdialysis study consists of 32 animals (e.g., control group and 3 drug treatment groups, with 8 animals per group). Sample sizes for our experiments are estimated from past experiments. Calculations typically show that we need group sizes of 8 to achieve the quality of results we need. On average we carry out 13-15 studies per year in rats, thus a total of 416-480 rats per year. Over the 5-year lifetime of the project licence, this equates to 2080-2400 rats. Mouse studies are typically carried out less frequently, thus the proposed estimated number of mice to be used is 350 (two studies per year).

A typical PK study consists of 20 rats (e.g., control group and 3 drug treatment groups, with 5 animals per group). Sample sizes for our experiments are estimated from past experiments. Calculations typically show that we need group sizes of 5 to achieve the quality of results we need. An average of 1- 2 studies per year (20-40 rats) are carried out, thus 100-200 rats during the 5-year lifetime of this project. For mice, an estimation of 1 study per year (20 mice), thus 100 mice in total estimated to be used over the lifetime of this licence.

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

We use the expertise of our statisticians who are able to perform power calculations to ensure that studies are suitably powered to detect changes. Similarly, the statisticians advise on experimental design to improve statistical power and reduce animal usage. Use

of the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) Experimental Design Assistant will also be considered.

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

Some surgically prepared animals may undergo re-use which will lead to a reduction in the total number of animals used across the lifetime of the licence. Blood or tissue samples will be collected from animals that have undergone surgery, where appropriate, to be use in further analyses to reduce the total number of animals used.

Refinement

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

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

Rats and mice will be used since their central nervous systems are well documented and they are the lowest form of mammal that can provide meaningful neurochemical, behavioural and pharmacokinetic data about humans. Occasionally, genetically altered animals may be used to model specific CNS disorders and the majority of these are likely to be mouse models. Animals with harmful phenotypes will not be tested and it is expected that the mutations will be well known.

The technique of microdialysis detailed in this project licence is widely used and has been used by the group for over 20 years. The technique allows changes in the levels of chemicals in the brain to be looked at. The microdialysis probe is made up of a fine metal tube with a very fine membrane on the end implanted in the brain, through this is pumped artificial cerebral spinal fluid (aCSF), that is the same composition as the fluid found in the brain, at a very slow rate, usually at 1 - 5 µl/min. Chemicals small enough to fit through the holes in the membrane will naturally diffuse across the membrane into the aCSF along their concentration gradient (i.e., lots in the brain and none in the probe so they will move from the brain into the probe to try and balance the levels). These chemicals along with the aCSF then continue out of the probe and are collected for analysis. This method therefore gives us a picture over time of how the levels of the chemicals of interest, such as neurotransmitters, change providing a measure of brain activity. In comparison to in vitro and ex vivo techniques, microdialysis is performed in the brains of intact, freely moving animals. Thus, the chemical changes in the specific brain region under investigation are subject to the normal control systems. Sequential samples are taken so it is possible to determine the profile of drug action on neurotransmitters and/or their metabolites, e.g., time of onset, magnitude of effect and duration of action; all of which are essential data in the evaluation of new centrally acting drugs/compounds.

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

Rats and mice will be used as their anatomy, physiology, behaviour and genetics has been well documented and they are the lowest form of mammal that can provide meaningful results that are translatable to humans.

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

Drug Administration

Vehicle and candidate compounds will be administered by the least severe route and administration will be performed by highly skilled staff, using appropriate dosing techniques, dose volumes and solutions (e.g., sterile solutions, appropriate pHs) to minimise any stress and discomfort to the animals. Appropriately sized needles will be used, and a separate needle will be used for each animal for systemic injection to maximise welfare and reduce the chance of inter-animal infections. Oral administration to rats will normally be by gavage using flexible catheters to minimise oesophageal trauma. Short oral dosing needles are typically used for mice which are likely to bite the flexible tubing. Refinements to oral administration of drugs such as training the animals to consume material voluntarily from a syringe have been considered but would not be practical and cannot be used as drugs have to be soluble in small dose volumes and palatable in sucrose or saccharin solution, which is not the case for the majority of drugs aimed at treating CNS disorders.

Where prior knowledge exists regarding administration of the test compound in vivo this will be used to optimise the choice of doses and route of administration wherever possible.

Surgery

In our extensive utilisation of microdialysis, we are constantly striving to improve the technique to reduce the likelihood of suffering in the animals and to improve our surgical and assay techniques to minimise the experimental failure rate. Aseptic technique will be applied at all times to reduce the risk of infection, based on the latest Home Office guidance and in keeping with the Laboratory Animal Science Association’s (LASA) latest guidelines. Previous knowledge gained through the modification of a mouthpiece which allows gaseous anaesthesia to be used on mice undergoing surgery was shared with other research groups, allowing them to refine their own surgeries.

To reduce stress in the animals prior to them undergoing surgery, they will be allowed to acclimatise to their environment for at least 6-7 days prior to surgery being undertaken. They will usually be weighed and handled for at least three days prior to surgery to familiarise them to handling and to provide information on their growth curve and general welfare.

The veterinary surgeon regularly observes our staff performing microdialysis surgery and inspects the animals post-surgery on a regular basis. Discussions are regularly held regarding aseptic technique, appropriate analgesia and other refinements, e.g., the fully implemented use of sterile drapes to cover the animal and reduce the risk of infection during surgery, as are used in human surgeries.

Analgesia

Careful consideration has been given to the need for pre/post-operative analgesia in conjunction with the Named Veterinary Surgeon (NVS). In microdialysis experiments, the subcutaneous lesion is made as small as possible to allow implantation of the microdialysis probe(s). However, implantation of a catheter and subsequent exteriorisation behind the neck results in more considerable tissue damage. For all experiments animals will be treated pre/post-operatively following consultation with the NVS (e.g., with a non-steroidal anti-inflammatory drug such as carprofen). The time course of the analgesia will be considered to ensure that it is beneficial from the time the animal may first begin to experience pain. An animal will not be used in an experiment until it is in a suitable condition, e.g., not displaying signs of experiencing pain, stress or discomfort. Animals are monitored for behavioural signs of pain by our staff who have extensive experience of post-surgical animals, by the NVS and by the Named Animal Care and Welfare Officers (NACWOs). Observations are recorded on individual post-surgical records by our staff. Extensive discussions were held prior to the previous licence application regarding use of Grimace Scales to recognise pain and assess its severity in post-operative animals (https://nc3rs.org.uk/grimacescales). Whilst the benefits of such a system were recognised, it was understood that in the case of animals that have undergone cranial implants, the facial features can appear altered due to the implant in the skull, unlike a non-surgical animal, making this scoring system less appropriate to adopt.

Housing

Prior to surgery it is expected that animals will be group housed. During the actual neurochemical sampling experiments, they will be housed individually in the dialysis bowls due to the nature of the equipment used. The bowls are made of clear perspex allowing the animals to see movement of their neighbours through the bowl walls. The bowls also allow auditory and olfactory communication between animals. Appropriate environmental enrichment will be provided, e.g., chew sticks.

In instances where animals will be returned to home cages following surgery, the cages used will be specifically chosen to reduce the risk of harm to the animals. For example, high top cages are used so that there is reduced likelihood of animals damaging their cranial implant by making contact with cage lid. Based on our extensive experience with post-surgical animals, it has been necessary to singly- house animals which have been returned to home cages following recovery from cranial implants. This is due to the high incidence of animals chewing on their cage mates' implants and resulting in the destruction of the implants. This will be reassessed if changes are made to the type of implants used, but currently it is anticipated that animals will be singly housed in home cages following surgery. The recommendations on housing by the NC3Rs will be taken into account, where the experimental procedure permits. Food and water will be available at all times and animals will be given increased environmental enrichment beyond the standard enrichment supplied (e.g., extra toys to play with).

They will also be handled regularly to provide the social contact they need as well as it being part of routine checks on their health and status of their implant.

Blood sampling

Heated chambers may be used to aid blood sampling from the tail vein. The use of a topical local anaesthetic to reduce any pain and discomfort during blood sampling will be considered. Temporary cannulation will typically be used in rats to avoid multiple needle

entries over short periods of time. Limits on volumes of blood samples will be taken from the NC3Rs guidelines.

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

We will follow guidance outlined in the PREPARE and ARRIVE guidelines for experimental planning and publication of data produced during this project.

We will also follow guidance issued by the Home Office, LASA, NC3Rs and RSPCA on animal re-use (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/470 008/Use Keeping_Alive_and_Re-use_Advice_Note.pdf), blood sampling (https://www.nc3rs.org.uk/microsampling and https://www.nc3rs.org.uk/general-principles), substance administration and needle use (https://www.nc3rs.org.uk/news/re-use-needles-indicator-culture-care, https://www.rspca.org.uk/webContent/staticImages/Downloads/AdministrationOfSubstances.pdf and http://www.procedureswithcare.org.uk/ASMS2012.pdf), aseptic technique (http://www.lasa.co.uk/wp- content/uploads/2017/04/Aseptic-surgery-final.pdf and https://researchanimaltraining.com/article- categories/aseptic-technique/), and housing (https://www.nc3rs.org.uk/3rs-resources/housing-and- husbandry/rodents).

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

We will keep abreast of the NC3Rs website for any updates as well as following any guidance or information passed on by our Named Information Officer (NIO). Alongside this we will attend any relevant seminars and webinars produced by groups such as NC3Rs and Responsible Research in

Practice. We will also keep up to date with the literature of advancements in the methods outlined in this licence and implement improvements to our working practices where practicable.

8. The effect of anthropogenic changes on avian biological rhythms and health

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

circadian rhythms, phenology, birds, urbanisation, light pollutio

Animal types	Life stages
Blackbird (Turdus merula)	juvenile, adult, neonate, embryo

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to understand the effects of urbanisation and human-induced land use changes on the behaviour, physiological health and fitness of wild birds.

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

Why is it important to undertake this work?

Knowing how birds cope with environmental change, and how their bodies meet this challenge, can inform us about consequences of environmental pollution, disruption of

natural diets and body clocks. In addition to gains in science, conservation concerns can also be addressed by this basic research on avian behaviour and physiology. One example is the way current climate change affects the progress of the growing season. Birds and other organisms already pay a cost for such changes because they time many of their activities by biological clocks that had evolved under natural conditions prior to major, man-made change. In animal welfare and also in conservation, there is need for remedies against disruption of biological clocks.

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

Outputs of this project will be:

1) the publication of research papers in peer-reviewed scientific journals

2) the publication of guidelines for the mitigation of the consequences that urban-specific environmental stressors such as light pollution, traffic noise and provision of food in bird feeders have on wild birds

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

The following stakeholders will benefit from the output of this research:

Applied academic researchers: the academic community will benefit from this research because we will publish peer-reviewed journal articles on the effects of urbanisation and land-use change on the health and fitness of wild birds, and the potential consequences for population persistence and biodiversity.

Policy-making sector: policy makers will benefit because they will be informed on the best practices for managing public urban green spaces for the persistence of wild bird populations.

The general public will benefit because we will raise awareness on the best practices to manage private green spaces for the persistence of wild bird populations.

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

Applied academic researchers: To engage the researchers working in these fields of applied research we will attend conferences and we will publish results in open-access journals. More specifically, we will present the results of the proposed project in high attendance, national and international conferences that will ensure the increased impact on research community.

Policy-making sector: The project findings will be relevant to landscape gardeners and planners (for instance city councils), lighting engineers and lighting industries. We will plan different activities to disseminate our findings to key relevant stakeholders and initiate partnerships that -with appropriate follow-on funding- will render our findings applicable to industrial and policy-making purposes. We regularly attend meetings with the local council in relation to proposed changes in planning for public green spaces, for instance the management of vegetation and the installation of new lights in city parks.

Impact pathways to the general public: We will actively inform the public about our research project through two main types of outreach activities.

First, we will liaise with the Public Engagement Group in Science, Technology, Engineering, Mathematics and Medicine (STEMM) of our university. This group organizes several annual science outreach events, including the STEMM Ambassador programme, and will help organize our participation on events and visits to local schools for engaging the public with our findings through presentations and educational activities. We will take part in Café Scientifique meetings, joining scientists and public in an informal setting, and in local and national science festivals.

Second, we will foster this public interest in science by open-access publications and by press releases through our local Media Relations Team. We have substantial media experience, and our work was featured on prominent media channels including BBC News, BBC Nature, BBC Springwatch, Huffington Post, The Independent, The Guardian, National Public Radio (US). We will build on this interest by following up on invitations by journalists to update them on new 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.

I plan to use common birds that are found in and around towns and cities in the UK. None of the proposed species are of conservation concern based on the assessments of the International Union for Conservation of Nature (IUCN), but some are of amber (willow warbler, song thrush) and red (greenfinch) concern for the Royal Society for the Protection of Birds (RSPB). All these species have healthy populations worldwide, but in the UK they have been declining locally or nationally in recent years, because of various anthropogenic factors such as urbanisaton, climate change and novel infectious diseases, often spread at bird feeding stations. It is important to understand what the drivers of change in health and fitness of these species are, and what we can do about it. My propose research aims at filling these gaps. I plan to study both young (nestlings) and adult birds, as the pressures and challenges that these different life stages may face because of environmental change can be different.

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

In the field, birds will be captured with standard ornithological techniques and released shortly after capture. Birds will be marked with unique aluminium rings from the British Trust for Ornithology, and a blood sample will be drawn from them for genetic and physiological analysis that are needed to assess kin relatedness, sex and health biomarkers. In some instances birds will undergo further procedures in the field, such as the collection of feather and faecal samples. All these procedures are brief (less than 5 minutes in duration). Sometimes birds might be tagged with external devices (eg GPS loggers), or exposed to the manipulation of their external environment (eg at their nest) in order to test specific questions about how certain environmental factors such as light pollution, noise or altered diet may influence behaviour and health. These experiments are longer in duration, from 2-4 weeks (manipulation of nest environment) to six months (GPS

tagging). Each individual bird will not undergo more than seven different procedures during its lifetime (although some of these might be repeated, such as capture and blood sampling).

In captivity, we will use captive-bred bird species (Japanese quail, Zebra finch) to conduct experiments tailored to understand short-and long-term effects of manipulation of urban-related environmental factors (light, noise, diet) on birds' physology and welfare. Birds will undergo very similar procedures to those applied to wild birds (except for GPS tagging), and thus will experience similar harms. These experiments will last up to six months.

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

All proposed procedures are of mild severity, but their combination might result in moderate severity. Capture, restrain and blood sampling might induce short-term effects (~ six hours), such as weight loss. Radio and GPS tagging are established techniques in field ornithology and will be further regulated by specific licences which we have already obtained from the British Trust for Ornithology. The manipulation of the external environment (light, noise, food availability) will be constrained within the range of environmental conditions that animals experience in their natural (rural or urban) environment. It might induce short-term effects such as weight loss (approximately 15 % of initial weight), lasting for a few days, but previous experiments did not suggest any substantial effect on individual long-term fitness and population growth.

For captive (indoor cages) experiments with zebra finches and quails, we expect limited adverse effects as all individuals used in our procedures will be originated from captive bred stocks. They will therefore be born in captivity and be used to living in cages. Both species are model species for captive laboratory studies on birds. Nevertheless, they might suffer from the same mild short-term effects related to capture and sampling, as specified for the wild birds above.

Expected severity categories and the 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 field procedures are considered mild, but the combination of GPS/radiotagging and blood sampling might be considered moderate, especially for the smaller species. This combination, therefore, will only be done on the blackbirds (2 % of total birds caught in the wild).

Most captive procedures on captive-bred birds (quails and zebrafinches) are considered mild, but temporary shifts into moderate severity can happen and can be treated and reversed to mild harm within a reasonable time.

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

- Killed
- Kept alive

Replacement

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

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

The proposed project strives to understand how animals respond to human-induced changes of the environment. This cannot be achieved without studying wild animals. Birds are an ideal study group because of their well-known ecology, and because they are large enough to carry miniature, wireless transmitters, which allow collection of data without a major burden to the animals. This technique replaces the need for recapture of the birds. The bird species that I intend to use are abundant, not endangered, and have been studied very successfully for a long time using similar methods tools to those I propose here.

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

This project is about wild animals and their ecology, I have considered using already available data published in previous studies, for instance by means of meta-analysis.

Why were they not suitable?

There is still so much that is still unknown about how human-induced environmental change affects wildlife that the data available is limited.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 wild-caught species possess ample genetic variation, and thus large sample sizes are needed to detect effects. I have used previous studies, including my own, to infer the amount of animals that would be needed for specific research questions and the relevant experiments that will need to be design to address such questions.

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

I have performed power analysis, consulted the designs and results of previous similar studies, and discussed the experiments I intend to perform for this project with more experienced biostatistician colleagues.

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 make the most efficient use of samples that I collect from the birds, to reduce the need to re-use the same animal, or to use more animals. For example, I will keep a stock of DNA for future investigations, which will allow me to answer upcoming questions without a need to resample more birds. For blood-sampling, I will reduce the amount taken to the minimum that is necessary to acquire reliable data. Furthermore, I will work closely with colleagues who carry out related research, so that information and samples will be shared between our teams.

Refinement

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

Which animal models and methods 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 proposed project involves birds because we know so much about their behaviour and ecology. Therefore, I can test well thought-out hypotheses based on previous data/information, using an adequate number of animals that is likely to produce robust data. Whenever possible, I will work on birds in the wild where I can perform procedures on site. This will greatly decrease the stress, and birds can be quickly returned to their natural environment. I will use the mildest possible procedures to collect behavioural, genetic and physiological data, such as using video-cameras, blood sampling, and biotelemetry, all established techniques in field ornithology. By using RFID tags (commonly used for pets) attached to rings and wireless telemetry, I can monitor birds remotely. I deploy an antenna in the nest cavity or on a tree, which then records identity, activity, body temperature or visiting rates of birds. These telemetry procedures will eliminate the need to recapture and handle a bird, thus minimising subsequent disturbance and while providing large sample sizes from relatively few birds. Moreover, I will use quails and zebra finches for captive cage experiment, to minimise stress impose on wild- caught birds when confined with small laboratory cages.

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

This is an ecological project and thus there is a need to study animals in their natural environment whenever possible. The need to tag and track the animals exclude the possibility to use less sentient species such as insects because they are too small to carry any tag device.

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

All animals that will be caught will be marked with unique identification rings and we use the recapture rate of known individuals as a measure of survival and lifespan. As not all of the marked individuals will undergo the same type and number of proposed procedures, there will be scope to compare the survival rate of, for example, birds that will only be blood sampled vs birds that will be blood sampled and GPS tagged, which can inform us on the welfare cost of GPS tagging for the marked animals.

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

https://www.nc3rs.org.uk/

https://www.ukri.org/about-us/mrc/our-policies-and-standards/research/research-involving-animals/3rs/ https://science.rspca.org.uk/sciencegroup/researchanimals/implementing3rs

https://twitter.com/NC3Rs

https://www.bto.org/our-science/projects/nest-record-scheme/taking-part/coc

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

I will be involved with the local AWERB committee and will regular sit in review meetings for other project licences. The committee also regularly sends emails about news and events that have to do with the 3Rs and with regulations of animal experiments in the UK.

9. Metabolism, Kinetic and Residue Studies with Chemicals in Domestic Livestock

Project duration

5 years 0 months

Project purpose

- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Agrochemicals, Chemicals, Livestock species, Pharmacokinetics

Animal types	Life stages
Cattle	juvenile, adult
Sheep	juvenile, adult
Goats	juvenile, adult
Pigs	adult, juvenile
Minipigs	juvenile, adult
Chicken	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 uses domestic animal species, which for the purpose of this project are defined as those species generally regarded as farm livestock, i.e. cattle, pigs and minipigs, sheep, goats and chickens.

This licence will investigate the Pharmacokinetics (PK), Pharmacodynamics (PD), Absorption, Distribution, Metabolism and Excretion (ADME) and Residue/biodistribution of agrochemicals/crop protection products; and other substances/entities for which regulatory safety assessment is required may be evaluated, including food additives or foodstuffs, or industrial chemicals. Chemicals designated as household products or cosmetics, or their ingredients, will not be tested under this project licence.

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

Why is it important to undertake this work?

Governments require (and the public expects) that substances we are exposed to are safe or that their potential hazards are well understood and documented. Before potential new chemicals are exposed to consumers, their safety must be evaluated. This is mandated by law to ensure public safety.

The data generated from the studies performed under this project will be used to inform decision- making processes on substances under development and, where appropriate, to satisfy governmental regulatory requirements necessary to gain marketing authorisation or product registration.

This safety assessment is of immense importance along with other non-rodent and non-animal studies in demonstrating to governments and the public the safety of these substances or highlighting their known hazards and safe handling.

New chemicals/agrochemicals have the potential to increase or protect food production while minimising safety risks to consumers and/or adverse effects on the environment. Before potential new chemicals/agrochemicals are used in the environment, their safety must be evaluated. This is mandated by law to ensure public safety.

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

This project licence authorises the conduct of in vivo pharmacokinetic/safety studies in target animal species to evaluate existing chemicals and novel and currently registered substances in terms of pharmacokinetics/pharmacodynamics, absorption, distribution, metabolism, excretion, residue kinetics and safety. This licence only authorises the testing of non-pharmaceuticals.

The overall benefit of this project is that it generates high quality data that is acceptable to regulatory authorities and support these submissions and enables internal decision making within our clients' organisations. This project will also ensure that chemicals that the general population are exposed to are safe.

Supporting studies, including preliminary studies and candidate selection, will enable appropriate dose selection and appropriately focussed observations and investigations in definitive regulatory studies.

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

Our customers will benefit, as the data we generate will allow them to progress their substances under development and, where appropriate, to satisfy governmental regulatory requirements necessary to gain marketing authorisation.

The project aims to test non-pharmaceuticals (agrochemicals, biocides, food additives /foodstuffs, ingredients of house-hold chemicals (where legislation allows) and industrial chemicals) in livestock species.

The studies ensure that non-pharmaceuticals such as agrochemicals and industrial chemicals (an occasionally other substances like food additives) that the human population are exposed to during their lives are safe or that their hazards are known so that they can be handled safely.

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

The work will be shared with customers who will use it to determine their future strategy, or for submission in documents required by regulatory authorities. Whilst we have no direct control over what happens to the data after we have shared it, we trust from information given to us that it is used for, or to support, regulatory purposes (e.g. to show that a certain chemical is safe for human exposure).

Where appropriate, we collaborate with our customers to share data we have produced in the form of Scientific publications that are in the public domain.

We are able to advise our customers on which studies are required in their development programme and on suitable study designs, based on our experience and on knowledge gained from previous post- registration feedback from customers and/or regulators, leading to focused and effective studies.

It is difficult to predict how the benefits of any work done on this project will be seen in the future due to confidentiality issues. However, this work will contribute to the safety of chemicals that the public and animals are exposed to.

Species and numbers of animals expected to be used

- Cattle: 120
- Sheep: 40
- Goats: 50
- Pigs: 70
- Minipigs: 70
- 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.

This project uses domestic animal species, which for the purpose of this project are defined as those species generally regarded as farm livestock, i.e. cattle, pigs and minipigs, sheep, goats and chickens.

The species used are species which may come into contact with the substances to be tested under this licence. Most of these species would appear in the food chain, and it is important to understand the extent to which humans may be exposed to the types of substances tested (i.e. levels of residues of substances which may be ingested by humans, for example).

Most studies would be carried out in adult animals; juveniles would only be used for specific studies where necessary (for example where patterns of use involve juvenile animals in the human food chain).

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

Animals are dosed by the intended/likely route of exposure (for example oral administration, dermal or inhalation), and observed regularly to monitor appearance, behaviour and clinical health.

Typically, on this project, animals are dosed over a period of time with test substances, and usually sampled (e.g. blood or urine) before having tissues taken, after they have been humanely killed, for analytical investigations. Study durations are dependent on the specific regulatory test being performed. Some animals are left dose free for a period of time after dosing is complete to investigate effects of depuration.

Dosing of animals is commonly done orally, either using a flexible tube or by capsule, or sometimes by incorporation in food. Other common routes include inhalation (when animals are normally dosed via a face mask) or dermal to mimic alternative potential routes of exposure.

Blood samples are usually taken from easily accessible veins, for example, in the neck of large mammals or the wings of birds. Limits are set on how much blood we can take at once or, cumulatively, over a month in order to prevent adverse effects of excessive blood loss. If an exceptionally large blood sample is needed for study purposes, we would do this when the animal is anaesthetised and we would not let them recover consciousness.

Where possible, we try to take as many of the tissues and samples we need after all dosing had been completed and the animals have been humanely killed.

If we need to take a urine sample for analysis, we would put an animal into a special collection cage which is smaller than their normal cage. The animal can still move around.

Other more unusual tests might include taking small samples of tissue under general anaesthesia, collection/examination of body fluids such as semen, body temperature by rectal thermometer. A minimal degree of restraint or confinement may be required for some procedures. Where appropriate, positive reinforcement training (using treat rewards) is used to encourage co-operation in (and minimise any stress of) handling/procedures.

Some animals may be used on procedure on more than one occasion (re-use); such re-use is limited and strict criteria are applied, e.g. veterinary examination indicates that it is appropriate to do so.

Some animals may be returned to domestic livestock use, again subject to strict conditions, under the supervision of a veterinary surgeon.

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

When dosing an animal by injection or taking blood, the degree of pain or discomfort an animal feels is similar to what a patient would feel having an injection administered by a doctor.

Most animals confined temporarily in a urine collection cage will get used to their new cage within a short time and show no adverse effects; however, in a small number of cases, some animals may show a degree of stress or anxiety unless the confinement is discontinued.

Dosing with chemicals may cause adverse effects in some studies. Experience shows that the majority (~95%) of animals may show transient subtle to mild clinical signs. Moderate signs of adverse effects may be seen in some animals (~5%), usually in the higher dose groups. Lethality and/or severe effects are not study objectives in any of the protocols within this licence and are not expected to occur.

We observe our animals at least twice a day, and the people who do this know the signs when an animal is unwell. If an animal is unwell, we would check it more frequently, and consult vets and other senior animal care staff for advice and guidance in its care.

Most animals are expected to experience only mild, adverse effects such as slight weight loss during the course of the study. A small percentage of animals may show more significant adverse effects, such as more marked weight loss, reduced activity, vomiting or tremors. No animals would be expected to die or to suffer prolonged adverse effects as a result of the procedures, and where necessary early humane endpoints are applied, under veterinary guidance as necessary, to prevent this; such endpoints might include interventions to discontinue dosing, or to provide supportive treatments, or if necessary to humanely kill 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)?

On the last project, about 95% of animals experienced mild severity, and around 5% of animals were classified as having experienced moderate severity. The moderate severities in the last project were either due to treatment-related signs of moderate severity (mostly in preliminary studies) or because a surgical procedure, e.g. cannulation, was involved.

A distribution between ‘mild’ and ‘moderate’ severities similar to those in the last project are anticipated.

All protocols on this licence are classified Mild or Moderate only, there is no intention to perform any procedures that are Severe in nature.

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

- Killed
- Kept alive
- 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?

There is currently no regulatory and scientifically acceptable alternative to the use of animals in these studies. These studies are run to satisfy the legal and regulatory requirements of governments around the world to ensure chemicals that humans may be exposed to (e.g. in the food chain) are safe for humans. These tests are very specific as to what they require in terms of testing in animals to ensure this.

We maintain a constant awareness of regulatory guidance and ensure that where non-animal methods exist which fulfil the regulatory requirement, they are used in preference to animal studies.

The regulatory requirements are mainly for UK/EU regulators, but occasionally other regulators in other countries like the US for example. If the requirements for these non-UK/EU tests are over and above the requirements for a UK/EU regulator, or the test required is more severe, then we consult the Home Office to ask for prospective authority to run such tests.

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

We maintain a constant awareness of regulatory guidance and ensure that where non-animal methods exist which fulfil the regulatory requirement, they are used in preference to animal studies. Currently, however, there are no other non-animal alternatives for the work being undertaken on this project. The regulations we are following will not allow safety decisions to be made on non-animal systems alone.

In vitro and in silico methods (test tube or computer work not using animals) are used in combination with animal studies to inform study designs and assist in understanding of potential ADME properties but cannot yet replace in vivo (animal) studies, and the complexity of a whole-body system.

Why were they not suitable?

Although there are test tube tests that can model some parts of how chemicals get into our bodies, and how our body deals with them, and can identify undesirable effects, for example, there is no series of test tube tests that brings all these complex processes together interactively, as in animals and humans.

That is why we need to test chemicals in animals; they have similar physiology and chemical processes to those in humans, and such testing gives a good indication of effects in humans exposed to the chemicals under evaluation (e.g. in the food chain).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 we have used are based on figures of previous usage from previous projects, or a projection thereof (based on estimated incidence) based on requests received from customers in the past.

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

Studies are designed to provide maximal data and statistical power (where appropriate) from the minimum number of animals considering that it is better to increase the number of animals used to achieve the objective than to use too few animals and risk having to repeat the study.

For regulatory studies, guidelines require the number of groups and animals per group to be adequate to clearly demonstrate the presence or absence of an effect of the test substance; core study designs are based on international guidelines where these exist. Otherwise, reference is made to standard study designs with input from the Department of Statistics, where appropriate, to identify the optimum number balancing the need to achieve study objectives while avoiding excessive animal use. These internal designs are reviewed and updated in line with changing external guidelines and internal refinements that either minimise numbers or reduce severity.

Whenever possible, common species of animals are used such that a large amount of control background data is available. This reduces the need for large control groups.

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

We will try to get as many outputs as we can from a single animal where possible, without adversely affecting its welfare. So, if we need to take several different samples, for example, we will often do that in the same animal, rather than using separate ones, when possible.

Before our main studies, we may use smaller groups of animals where applicable to get an idea of the doses appropriate to use for the main studies. These preliminary studies, where they are appropriate, are important as they give us confidence that the doses we are using are correct prior to testing them in larger groups of animals as required by global regulators.

Refinement

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

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

This project uses domestic animal species, which for the purpose of this project are defined as those species generally regarded as farm livestock, i.e. cattle, pigs and minipigs, sheep, goats and chickens.

The models we use are the least invasive procedures, for the least amount of time necessary to get the information we need. They are carried out using standard and recognised techniques by fully trained staff. We also have veterinary clinicians on hand for clinical advice, to assist with technical procedures/anaesthesia etc. where necessary, and to provide general advice on animal welfare

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

In order to fulfil the scientific objectives of the studies, and as a requirement of the regulations under which the studies are mandated, studies must be performed using animals of the same species, type, age and class as those that will be exposed to the test substances under normal conditions of use after approval and marketing, and will be involved in pathways resulting in potential human exposure.

Most of these studies require repeat dosing for days or weeks, to fulfil the study objectives, so it is not practical to perform them under terminal anaesthesia.

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

During dosing and restraint, animals are constantly and closely observed for signs of distress. If equipment is used to enable us to achieve the scientific aims of the study (e.g. confinement in a metabolism cage for urine collection), then we would habituate animals to the equipment prior to study use. Most animals habituate well to this equipment, but if they don't (rare) we remove them from the study.

If we have to repeatedly inject animals or withdraw blood using a needle and syringe, we would choose different sites to do this where possible to minimise local adverse effects.Where appropriate we place temporary cannulas in blood vessels to reduce the number of needle punctures necessary. If we can take blood samples when an animal is anaesthetised, then we do so. All personnel performing these procedures are trained to a high standard to minimise adverse effects.

All procedures are subject to ongoing assessment and technique improvement, and we participate in cross-company working parties on best practice. Animals are regularly reviewed for general health and veterinary staff are on call at all times to assess any adverse events and provide supportive care and treatment as appropriate.

Refinements to improve the animals’ experience include but are not limited to group housing, environmental enrichment, including novel food treats, human interaction, acclimatisation and training to procedures, and calming measures such as stroking/gentle talking are used to help animals have a better experience of restraint. We have dedicated working groups on animal welfare for each species with a permanent brief to identify potential measures to improve animal welfare, and to trial such measures.

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

OECD Guidelines for the Testing of Chemicals: OECD 503 (2007), Metabolism in Livestock; OECD 505 (2007), Residues in Livestock

Residue Chemistry Test Guidelines: OPPTS 860.1300 Nature of the Residue - Plants, Livestock [EPA 712-C-96-172]

Residue Chemistry Test Guidelines: OPPTS 860.1480 Meat/Milk/Poultry/Eggs [EPA 712–C–96–182]

Diehl et al. A good practice guide to the administration of substances and removal of blood, including routes and volumes. Journal of Applied Toxicology: 21, 15-23 (2001).

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

This will be achieved by regular discussions with our Named Information Officer, colleagues in Animal Technology, and by attending appropriate training courses and conferences, or getting feedback from such events.

10. Pre-clinical evaluation of therapeutic agents and combinations in orthotopic oncology models

Project duration

5 years 0 months

Project purpose

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

Key words

cancer, mouse models, orthotopic, pre-clinical

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 primary aims of this project are:

(i) To develop patient relevant pre-clinical mouse models.

(ii) Use mouse models to evaluate therapeutic agents (mainly anti-cancer) to support progression of effective treatments to human trials, ultimately resulting in validated clinical 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?

Cancer is a broad group of diseases involving unregulated and uncontrolled cell growth that leads to the development of malignant tumours that can invade nearby parts of the body and/or spread to distant organs. There are over 200 different known types of human cancer, which can be treated with different approaches including surgery, chemotherapy, radiotherapy, targeted agents and more recently immunotherapy. Cancer remains a huge health problem globally and specifically within the UK results in just over one in four deaths (2021) and accounts for 167,142 deaths per annum (2017-2019). The latest statistics on cancer mortality from Cancer Research UK (http://www.cancerresearchuk.org) confirm that the major killers remain the solid epithelial cancer types where death is linked to the spread of cancer to other organs such as brain, bone, liver and lungs.

Although several anti-cancer drugs enter clinical trials each year, only 5% of new anti-cancer agents showing preclinical activity go on to be licensed after successful Phase III trials. This is because the pre-clinical models used to mimic cancer do not accurately reflect each of the individual factors that contribute to disease for example the structure of the organ that the cancer originates in, the blood supply to the organ and the immune system influences on the cancer. For these reasons, pre-clinical animal models often lead to unacceptably high failure rates in human clinical trials with around 77% of 800 anti-cancer drugs failing early on in clinic, mainly due to the poor response to the anti-cancer agents in the patients tested.

This data highlights the growing need for Pharmaceutical, Biotech and academia to develop more disease and patient relevant pre-clinical cancer models that better mimic what happens during the growth and progression of cancer in humans, before the new generation of anti-cancer agents enters clinic. This is especially vital with the increased investment into this field and the growing catalogue of new immunotherapeutics, combination treatments and re-purposing of drug candidates.

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

In order to benefit the clients/collaborators who develop the anticancer agents and ultimately patients who are treated with these agents, the development of pre-clinical cancer models that exhibit greater patient relevance by implanting them in relevant organs will allow these novel agents to be tested in more relevant conditions where environmental factors such as blood supply, spatial arrangement, interaction with supporting cells and structures will be better represented. These models require expertise in surgery as well as generating the cells that emit light and then applying the imaging technology to capture the right data and analysis, which is not readily available in most institutions and companies. These models will enable decision on moving programmes forward into clinical trials or in some cases this may result in a specific anticancer programme being cancelled which may seem a negative benefit, but identifying anticancer agents that are either ineffective or unsuitable for further development can be considered a positive benefit in the longer term as it prevents the unnecessary progression of ineffective therapies to early phase clinical trials and allows the redirection of resources and patients to other projects. Once validated, all models are added to the proprietary databases; access to which is free to all users, as well as abstract submission to national and international scientific conferences.

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

We are a contract research organization (CRO) that develops/generates and validates animal models for use in client-commissioned studies to evaluate candidate anti-cancer therapies. The company is engaged in this research by refining and developing pre-clinical

models that exhibit greater patient relevance and performing studies with these models in order to support the clients/collaborators we work with who are actively developing new agents and regimens to target cancer. Having established a growing global customer base for the pre-clinical evaluation of anti-cancer agents, we will continue to provide our centralised service to a large number of clients who either do not have the facilities (e.g. virtual Biotech companies), relevant cancer models, expertise or capacity to carry out studies.

Customers of this type have a preference to outsource this work to a 3rd party service provider where there is a high level of experience, knowledge and clinically relevant models within facilities that are appropriate for such work. In addition to this, our service offering allows customers to focus on other components of their research thus helping them realise benefits more quickly by increasing efficiency and productivity.

The immediate output of this project will be the development of orthotopic models of cancer which are relevant to the clinic as the cancer is implanted in the site of origin. Once these animal models are validated they are immediately available for client services and all animal models that are developed in house are added to our publicly accessible databases. Access to these databases is free for to all users, so one of the immediate benefits of the animal model development process is that model data, including growth and response to known treatments, key features of the tumour, is freely available to the scientific community, which makes these databases extremely powerful tools for research.

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

Information around new animal models, as well as factsheets and application notes on specific research areas, are freely available to the general scientific community through focused marketing activities including free symposiums and client presentations.

- As a company are very proactive in attendance at relevant national and international scientific conferences, and the company regularly participates in these conferences. Outcome of research is actively shared with the global scientific community through abstract submission at these conferences. In addition, research is submitted to peer-reviewed journals, where possible, in order to disseminate findings. In some cases, cost sharing development of new animal models for clients, or refinements of existing animal models, enable further research and development of more clinically-relevant models. This allows the new models to be available to wider scientific community and findings disseminated.

Species and numbers of animals expected to be used

- Mice: Mice: 20,300 across all 10 protocols

Predicted harms

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

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

The architecture of mammals is required to accurately model tumour development and spread to relevant organs and mice are the lowest order species in which tumour

development can be modelled and evaluated. Models where the cancer is grown in the organ of origin are known to better model cancer in patients with respect to various criteria; they form a single area of tumour, as would be found in patients, but they also have the required factors to enable the cancer to spread via the blood stream and/or lymphatic system.

Mice will be used for this project as they are the lowest species of animal that allow the modelling of human cancer, and offer the opportunity for genetic manipulation to generate specific models relevant to human cancer.

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

During an ~30 minute timeframe, cancer cells will be injected into the site of origin of mice (e.g. prostate, bladder, pancreas, lung, heart, liver) using either a surgical method, where a small incision is made, or via ultrasound-guided injection, both under anaesthesia. These animals will experience some discomfort after surgery and some mild to moderate pain which will be minimized with analgesics and animals will be very closely monitored. The growth of the tumour within the animals will then be monitored, up to three times a week, under anaesthesia by visualization of the tumour mass if the tumour cells have a specific bioluminescent/fluorescent label. Animals with tumours that did not have this label will be carefully monitored every day for clinical signs associated with tumour growth. Animals may consume supplemented diet and/or water which is not expected to cause distress but may sometimes result in body weight loss if the diet is unpalatable. If the animals experience >15% body weight loss they may be placed back onto normal diet. Animals may also have food removed for up to 16 hours.

Substances may be administered to the animals using standard routes (e.g. intravenous, subcutaneous, intraperitoneal, orally) and typically, animals will experience mild, transient pain but no lasting harm from administration. Provision of supporting tolerability data or acute phase tolerability studies means that the frequency of treatment-related adverse effects is uncommon in these studies. If the administration of substances is required for prolonged periods, a separate slow-release drug device may also be implanted under the skin of the animal. Animals may have blood, urine or faecal samples taken (urine and/or faeces obtained via non-invasive methods) and this will lead to mild and transient discomfort. At the end of the project the animals may have final samples taken under non-recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and may experience mild distress and no pain.

In some studies where the tumours are sensitive to hormones to help the tumours grow, it may be necessary to supplement the animals with hormones using the most refined method (e.g. addition to the food and/or water, slow release implants under the animals skin). In other situations it may be necessary to perform studies where all of the influence of hormones are removed and for this we will use castrated animals from specific breeding establishments.

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

Mice will have surgery under anaesthesia to implant cancer cells within a specific organ, or to implant a device under the skin that can release substances slowly. The mice are expected to recover quickly from the procedures and will be given painkillers and post-

operative care. Animals will be pain scored for up to 3 days post-operative and any animals with a pain score of 1 or above on day 4 will be humanely killed.

The tumour growth in the organ will be monitored closely and if the tumour exceeds a pre-defined endpoint the animal will be humanely killed. If the tumour spreads beyond the organ of interest and leads to increased tumour growth at the metastatic site compared to the primary site, and/or the tumour impairs the normal function of the animal, the animal will be humanely killed. If any clinical signs are observed (e.g. hunched posture, subdued behaviour - responsive when provoked) the animal will be humanely killed if the animal does not return to normal clinical condition within 2 hours. If the animal is in a poor clinical condition and/or un-responsive they will be immediately humanely killed.

Surgical procedures and/or administration of substances may cause body weight loss and if this body weight loss exceeds 15% the animals may be placed on a dosing break. Mice with body weight loss of ≥18% will be humanely killed. Mice with body weight loss ≥15% will be monitored for a maximum of 2 further consecutive days for improvement in body weight. If body weight does not recover to <15% during this time the animal will be humanely killed.

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

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

95% of animals are likely to be classified as moderate severity as they will undergo surgical procedures to implant cancer cells/material. 5% of animals are expected to be mild severity as they will be used for simple dosing in tolerability studies. The animals used under mild severity will be non- tumour bearing and will not have undergone a surgical procedure.

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

- Killed

Replacement

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

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

In vitro / in silico methods may be useful to predict absorption, distribution, metabolism and elimination of new anti-cancer drugs properties, as well as target engagement and pharmacology assessment of a well-defined compound series. This can be used as an initial screen to help to prioritize compounds for further evaluation in animals, however in vitro / in silico methods are unable to predict accurately the full in vivo effects that a novel compound may have when administered to animals in terms of any potential off target effects or toxicity. The tumour/host interaction is complex with many different cell types and systems (e.g. the blood and lymphatic systems) interacting and communicating with each other. Currently there are no in vitro systems that are able to replicate these complex processes.

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

In vitro methodologies have replaced animal use to a degree in cancer research, particularly in the development of screening assays to refine compound selection, target identification, off-target toxicity or toxicity versus normal cell lines. These in vitro assays can certainly be used to guide and refine the steps prior to moving into in vivo studies, and thus minimize subsequent animal use. Our company is actively working in the field of in vitro assays and we are developing cutting edge technologies to enhance the in vitro studies we offer which recently include the development of organoid assays, offering customized cell screening assays of up to 250 cell lines and the use of high content imaging to allow precise drug/tumour interactions to be visualized.

Why were they not suitable?

Although in vitro tumour growth assays are routine within our company, and can facilitate the identification of suitable cell lines or lead candidates, there is still a requirement to use animals for the project as the 2D and 3D assays still do not optimally mimic all interactions between cells and tissues in vivo, therefore in vivo models are still required prior to progression of new drugs into human clinical trials.

Research and development of anti-cancer agents involves a multi-step process in which in vivo studies form an integral part of the regulatory process linked to the approval of new drugs. Drug products not previously authorized for human use must undergo a submission application process before progressing to human trials, and applicants are expected to support that process with in vivo pharmacology as part of the submission.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 have estimated to use is based on several factors including:

- the client demand for the model and how this has changed from previous years
- assessing the change in focus based on published articles
- where research council funding drives are directed, to ensure that we can meet demand for specific models in the relevant areas

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

The use of in vitro studies can be used to identify lead compounds, evaluate dose ranges confirming target modulation/expression and relative off-target toxicity which can be used

to inform on relevant doses for use in pharmacology based studies and pilot toxicity studies.

Study format will vary on a per project basis, and may range from 2-12 groups; test agents may be blinded dependent on the particular project requirements. Studies will include at least one control arm, and control arms for both route and frequency, as well as changes in vehicle composition, will be employed where relevant. Where possible, the use of positive control groups (either a standard of care or an agent that induces significant growth inhibition) will be regularly employed to ensure the model is behaving consistently. For combination dosing, dosing should be balanced across all study groups, to ensure that this is represented in the vehicle groups.

Group numbers will be based on power analysis (supported by our dedicated statisticians in China) but typically range from 10-20 mice/group for efficacy testing of internal bioluminescence, variability tends to be higher due to absorption and reflection of bioluminescent signal by internal organs and tissues, therefore higher group number are employed to maintain study power.

The model development stage of this project will be used to determine statistical power to ensure the minimum number of mice are used in a study design, but with the ability to still achieve robust scientific endpoints. The use of imaging technologies can also reduce the number of animals required to generate study outcomes as model variation can be improved by eliminating mice which do not develop the disease appropriately or refining the model (e.g. using ultrasound-guided injection) so this can be minimized.

Guidance on experimental designs and methods of analysis of the results will be obtained from NC3Rs (https://www.nc3rs.org.uk/experimental-design) and by attendance at relevant online seminars that are being hosted by NC3R to ensure that we are keeping up to date with the most relevant design guidelines and data analysis.

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

The use of short-term tolerability assessments may be avoided if supporting data is already available in a relevant model (the correct animal strain, tumour model, compound batch and dosing route/regimen).

Pilot studies will be performed on any newly developed animal model, part of which will facilitate the identification of experimental variation and this will be used directly in powering studies to achieve statistical significance. The use of a structured committee approach to the decision on which models progress to the development stage, ensures that the process is both reflective of the scientific aims and conducted in a way that minimizes the use of animals to achieve the project aims. Study design will incorporate numerous controls both in statistical design, and the application of correct vehicle and dosing controls groups. Each study is governed by an experimental protocol and the generation, review and application of protocols in delivery of studies is governed by SOPs and underpinned by the QMS system. Protocols include a statement of the objective(s); a description of the experiment, covering such matters as the experimental treatments, the size of the experiment (number of groups, number of animals/group), and the experimental material; and an outline of the method of analysis.

Where possible, we will make appropriate arrangements to randomly assign animals to experimental groups and blind studies. Experiments will be planned, where possible, so they can be published in accordance with the NC3Rs' ARRIVE guidelines.

For efficacy study formats, candidate anti-cancer agents can typically be challenged in one of two scenarios:

1.Efficacy against established tumours: Tumour-bearing mice are randomised to control and treatment groups based on bioluminescent signal/ultrasound tumour volume measurement at the earliest time point that will deliver the scientific objective. For slow growing models a static phase may precede a growth phase which is progressive, and typically for these models dosing will start when the progressive growth phase has been identified. For models that use unlabelled cells, animals will be randomised into groups at a timepoint that would facilitate an adequate dosing window (2-3 weeks dosing window is aimed for) and randomisation would typically be based on body weight. Pilot studies will provide important information on the rate of tumorgenicity and growth profile to guide efficacy on overage, assignment and dosing schemes. Models of this type will usually have a higher overage to take into account differences in take rate and growth of tumours.

2.Tumour prevention: In these studies mice are randomised to control, and treatment groups based on body weight prior to tumour inoculation; the group numbers will typically be larger as there is no provision for removing outliers or animals without established tumours prior to commencement of treatment. Dosing will typically commence from up to 0-14 days prior to tumour inoculation and continue thereafter, with the pre-dosing duration driven by the agent/strategy under investigation e.g. administration of a blocking antibody may only be required on the day of implantation, whereas a bacteria preparation designed to increase immune response may require 14 days for complete colonization.

For studies looking at efficacy (Objective 2) with already established models, implantation of an excess of mice helps reduce overall total animal use on study by minimizing study variation at randomisation by allowing the statistical removal of outliers (e.g. Stem Leaf analysis; Minitab 16), inconsistent or non- growers, and animals displaying early clinical signs that may compromise study design and/or animal welfare e.g. early signs of tumour disseminating in non-target organs.

Where possible we will look to use high-frequency ultrasound guided injection as a method of tumour initiation. This has the following 3Rs advantages over traditional surgical approaches including:

- animals experience less adverse effects compared to initiation of tumours using traditional surgery

- the reproducibility of using ultrasound guided injection is much less varied that that observed with traditional surgery

- recovery time from surgery is far quicker using ultrasound guided injection compared to traditional surgery

Where relevant and available, the use of longitudinal imaging modalities, either bioluminescent and/or fluorescent, will be used to reduce the numbers of animals used. For example, for an orthotopic model whose internal tumour dimensions cannot be measured, tumour growth would normally have to be characterized by timed terminations

of multiple study groups. Using imaging technologies, this can be assessed in a single cohort of animals by utilizing multiple image points, longitudinally on the same cohort of study animals. Frequency of imaging sessions will be balanced with model duration to ensure that the growth is adequately assessed whilst minimizing the number of procedures carried out. The use of imaging technologies can also reduce the number of animals required to generate study outcomes as model variation can be improved by eliminating mice which do not develop the disease appropriately (e.g. the tumour fails to seed in the expected location) or refining the model so this is minimized.

Pilot model development studies performed under Objective 1 will aid in the determination of a suitable number of excess mice to implant to ensure that sufficient animals with a suitable tumour BLI/size range are recruited on to study. The excess will be under review to ensure that the most appropriate is used and this will be adjusted if deemed scientifically appropriate.

Refinement

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

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

For the generation of tumours from tumour cell lines grown in vitro, (wild-type, labelled or modified genetically), cells undergo a highly refined process (driven by SOPs, underpinned by the QMS) with numerous checkpoints and controls, to ensure only high quality viable aseptic cells are used.

When undertaking tolerability studies, the mouse strain selected will typically be consistent with the strain proposed for the follow-on efficacy study. However, where it is considered unlikely to impact on the scientific outcome of the study, the background strain of a mutant (immunocompromised) or genetically altered (GA) mouse strain may be employed to reduce the use of these types of animals.

The mice will have tumours implanted into the prostate, pancreas, liver, brain, heart, carotid artery, lung or bladder, which are more relevant to patients, but are more technically complex and most will require imaging (either bioluminescent and/or ultrasound) to track the growth of the tumour within the mouse.

For bioluminescent imaging (BLI), this is achieved by using tumour cell lines for implantation that are altered to emit light, which can then be captured by an imaging system specifically designed for small animals. For monitoring of tumour growth by ultrasound, there is no requirement for the cell line to be altered to emit light prior to implantation as the ultrasound is capable of detecting the presence of the tumour mass within the organ in which it was implanted. For some models e.g. orthotopic bladder models, the animals can also be imaged without the need for anaesthetic which is a refinement for the animal. The exception to this is when patient-derived xenograft (PDX) models are developed and validated; PDX tissue is difficult to bioluminescent/fluorescent

tag and therefore monitoring of tumour burden in these models is via the use of daily monitoring for the appearance of well-known and documented clinical signs and humane endpoints. Each model has its own clinical signs and defined humane endpoints, which are described in the individual protocols for each model.

For hormone dependent models (some oestrogen-dependent breast/ovarian models) hormone supplementation using the most refined method that results in consistent tumour growth for efficacy testing will be used. Alternative supplementation in diet and/or water has been tested and further refinements will be evaluated.

In preclinical studies in nude mice treatment with vaccinia virus results in the formation of pox-lesions (red papules) on the tail which is a typical symptoms of systemic infection by vaccinia virus 4-5 days post i.v. injection which improve 8-10 days later. Lesions will be a dry raised red papule, but if lesions appear wet or show signs of infection then the animal will be humanely killed. If the lesions do not show signs of improvement after 8 days from first appearance will be humanely killed. In addition the injection of the virus will be carried out towards the base to reduce the length of the tail exposed to the virus and reduce the frequency of the lesions. Pox-lesions may also appear on the feet and nose of the animal, animals will be humanely killed if these appear.

Where possible, any new orthotopic models will be validated using high frequency ultrasound-guided injection of the tumour cells to the specific organ. In our preliminary studies, e.g. pancreatic and liver ultrasound-guided injections, we have found a significant improvement (reduction) in both surgery time and recovery from the procedure in animals implanted via ultrasound-guided injection compared to traditional surgical implantation. In addition, animals experience less adverse effects compared to initiation of tumours using traditional surgery and the reproducibility of using ultrasound guided injection is much less varied that that observed with traditional surgery.

Generally the use of non-invasive longitudinal bioluminescent imaging and/or ultrasound guided measurements will be used to refine the methods used for all orthotopic cell line models. As well as minimizing animal suffering, it allows the opportunity for the determination of a statistically significant result ahead of a scheduled termination, thus potentially reducing the duration and use of regulated procedures on animals.

Organ-specific models are known to better model cancer in patients as tumour grows in the correct environment which facilitates spread to other organs as seen in the clinic and show a reduced response to chemotherapy therefore providing more relevant information on the drug. The use of imaging is also a refinement as data from the internal tumours can be captured in real time, provided additional data that wouldn’t normally be visible, only using animals that show tumour, and minimize animal suffering, as it allows the opportunity for the determination of a statistically significant result ahead of a scheduled killing of the mice, thus reducing the duration of model and regulated procedures.

For orthotopic prostate cancer models, tumour engraftment is established by a cell injection directly into the prostate following laparotomy. Depending on the specific cell line model under challenge, it typically takes ~1-2 weeks to establish tumours and experiments will normally use a continuous dosing regimen of 2-4 weeks. Pilot studies (Objective 1) will be used to further refine the model such as cell inoculation volume. For hormone dependent models e.g. LNCaP prostate cancer, hormone supplementation using the most refined method that results in consistent tumour growth for efficacy testing will be applied if needed. Alternative supplementation in diet has been tested and further refinements will

be evaluated. Primary humane endpoints have been identified typically as impingement of ureter or urethra function resulting in enlarged bladder and abdominal distension. For each cell line model an intervention measure of tumour-associated bioluminescence (TABL), tumour size (volume, area) measured by ultrasound and/or daily monitoring for the appearance of well-known and documented clinical signs (unlabelled cells e.g PDX material) will be determined during pilot studies, prior to the onset of any adverse effects.

For orthotopic pancreatic cancer models, tumour engraftment is established by either intrapancreatic cell injection following laparotomy or by visualization and inoculation using high frequency ultrasound- guided injection. Depending on the specific cell line model under challenge, it typically takes ~1 week to establish tumours and experiments will normally use a continuous dosing regimen of 2-4 weeks.

Pilot studies (Objective 1) will be used to further refine the model such as cell inoculation volume. Intervention measures of TABL and/or tumour size (volume, area) measured by ultrasound, or daily monitoring for the appearance of well-known and documented clinical signs (unlabelled cells e.g PDX material) will be determined during pilot studies, prior to the onset of any adverse effects. Typically for pancreatic cancer models the mean tumour area will not exceed 10.5mm2 as measured via ultrasound.

For orthotopic bladder cancer models, tumour engraftment is established either by intravesicular implantation to mimic superficial disease or directly into the bladder wall to model invasive disease. Pilot studies (Objective 1) will be used to refine the two models for cell inoculation volume and intervention measures using TABL prior to the onset of any adverse effects. Depending on the specific cell line and route, it typically takes ~1 week to establish tumours in the bladder and experiments will normally use a continuous dosing regimen of 2-4 weeks depending on the duration of the model (superficial tumour model will typically use 2-3 weeks of dosing compared to 3-4 weeks for invasive bladder wall model). Intervention measures of TABL and/or tumour size (volume, area) measured by ultrasound, or daily monitoring for the appearance of well-known and documented clinical signs (unlabelled cells e.g PDX material) will be determined during pilot studies, prior to the onset of any adverse effects. Typically for superficial bladder cancer models the mean tumour area will not exceed 26mm2 as measured via ultrasound.

For intracarotid models, the primary aim is for the injected tumour cells to spread and form primary tumours within the brain. To mimic the metastatic spread of primary tumours to the brain, cancer cells will be injected into the left common carotid artery of mice as opposed to the internal carotid artery. To facilitate this we recommend a number of refinements as guided by the literature: Increased incidences of metastatic deposits in the face, ears and facial skin have been reported when cells were injected via the internal carotid artery as the tumour cells were found to transit through the branches of the external carotid artery. In order to overcome these unwanted metastatic deposits the left common carotid artery will be ligated caudal to the injection site to block blood flow. The left external carotid artery will be also be ligated rostral to the common carotid artery injection site to prevent the movement of cancer cells via the external carotid artery. Undertaking this ligation procedure has been reported to significantly reduce the incidence rates of metastatic deposits in the face, ears and facial skin, whilst improving the migration and seeding of cancer cells towards the brain. For hormone dependent models (some oestrogen-dependent breast/ovarian models, and some androgen-dependent prostate models) hormone supplementation using the most refined method that results in consistent tumour growth will typically commence up to 2 weeks prior to implantation. The duration of the

model is dependent on the cell line used and can typically vary from 2-6 weeks. Primary humane endpoints for intracarotid models have been identified as ataxia and head-tilt, which typically present prior to further deterioration in clinical signs; thus for each cell line model, an intervention measure of TABL will be determined during the model development process, to guide scientific endpoint prior to the onset of clinical signs.

Furthermore, if tumours grow at off-target sites (face, ears etc.), animals will be humanely killed

Intracardiac administration of cells results in bone metastasis, which is a more refined model than direct injection into the bone as the circulating cells encounter the target organ e.g. the capillary beds of the bones, in the same way as circulating metastatic tumour cells arising from a primary tumour. In the capillary beds they are compelled to invade into the tissue, thus only the clone of the cell population having the required capabilities e.g. tropism conferred by possession of the bone metastasis gene expression signature, will survive and grow into a tumour. Direct injection into the bone introduces the cells directly into the site and does not model the extravasation and intravasation of cells into the bone site. Furthermore, direct injection may result in the mechanical disruption of the bone itself, which is not only aversive to the animal, but could also compromise the development of lytic lesions that are characteristic of many breast and prostate bone metastases. Our company have previously used this procedure to model prostate, breast and melanoma bone metastasis with humane endpoints optimised through pilot study model development (Objective 1) prior to efficacy testing (Objective 2) using TABL as a refinement. Further refinements such as injection technique have already been assessed by an external expert trainer. Furthermore, the injection into the left lumen of the heart avoids the pulmonary circuit, allowing a complete circuit of the body before the cells encounter the capillary beds of the lung. This allows the circulating cells to encounter the capillary beds of the long bones, in the same way as circulating metastatic tumour cells arising from a primary tumour. The use of juvenile mice (≤6 weeks) improves the tumour take-rate in the long bones (femur/tibia) as the growth plates typically fuse soon after this age range. Bioluminescent imaging is carried out immediately following inoculation, whilst still under general anaesthesia, which will confirm whether the injection has resulted in a full systemic expansion, or is limited to primarily lung engraftment (indicating injection into the right-side of the heart). Mice exhibiting the latter phenotype will be humanely killed prior to recovery. For hormone dependent models (some oestrogen-dependent breast/ovarian models, and some androgen-dependent prostate models) hormone supplementation using the most refined method that results in consistent tumour growth will typically commence up to 2 weeks prior to implantation. The duration of the model is dependent on the cell line used and can typically vary from 2-6 weeks. The primary humane endpoint for any cell line used in this bone metastasis model is progression of bone lesions; typically lesions in the long bones (fore and hind- limbs). This results in splayed limbs, partial or full paralysis of the limbs, ataxia, as well as discomfort/pain. For each cell line model, an intervention measure of TABL is derived during the model development (Objective 1) process at a point prior to the onset of these clinical signs e.g. >1x109 photons/second for MDA-MB-231 bone lesions. Because TABL informs on progression of bone tumours before evidence of pain, analgesia is not used to control pain, the mice will be terminated prior to the point at which pain is evidenced. Additionally, use of analgesics at this point could result in potential drug-drug interactions that may confound the scientific outcomes and/or result in additional toxicity.

For intrahepatic liver models, tumour engraftment is established by intrahepatic cell injection following laparotomy or using high frequency ultrasound-guided injection. Depending on the specific cell line model under challenge, it typically takes ~1-2 weeks to establish tumours and experiments will normally utilize use a continuous dosing regimen of 2-4 weeks with the dosing regimen. Refinements have already been made on this model using certain cell lines models to ensure tumour growth is established within the parenchyma of the liver and TABL used to assess tumour burden. Furthermore, on previously developed models we have already established that the mean tumour diameter will not exceed 10.5mm as measured via ultrasound. For new cell lines progressing through Objective 1, intervention measures of TABL and/or tumour size (volume, area) measured by ultrasound, or daily monitoring for the appearance of well-known and documented clinical signs (unlabelled cells e.g PDX material) will be determined during pilot studies, prior to the onset of any adverse effects.

Intracranial implantation presents a very different set of problems to that of other target organs due to access to the brain. We have already established and refined a number of brain models within our company. Modelling the tumour within the brain enables evaluation of a candidate drugs ability to cross the blood brain barrier (BBB) and also allows an avenue for direct dosing for test agents whose efficacy may be impaired by the BBB. Under this model, small proof-of-concept pilot studies may be carried out whereby dosing is achieved by administration directly into the brain or tumour site. Where multiple doses are required use of a specialized cannula will be implemented to reduce the number of invasive procedures as a refinement. For efficacy studies the typical duration of the model is ~3-5 weeks. Primary humane endpoints for intracranial models have been identified as body weight loss, ataxia and head-tilt, which typically present prior to further deterioration in clinical signs; thus for each cell line model, an intervention measure of TABL (e.g. >1x109 for U-87MG) can be determined during the model development process, to guide scientific endpoint prior to the onset of clinical signs. Where it is not possible to utilize BLI e.g. PDX models, the model will be characterised through staged terminations to allow full characterization of tumour burden with model duration and changes in clinical signs. Humane endpoints typically include changes in normal behaviour, deterioration of clinical signs and/or progressive body weight loss and the mice are humanely killed at this point. Persistent adverse clinical signs e.g. subdued behaviour patterns even when provoked, will result in animals being humanely killed, regardless of body weight measurement.

Intrathoracic lung implantation of bioluminescent tumour cells into the left lung results in contralateral lung metastasis followed by metastasis to bone, liver and brain. The primary humane endpoint for any cell line used in this lung model is progression of lung tumour, which results in changes in respiration (rate and depth), pale extremities or changes in behaviour that would indicate an increased respiratory burden. An intervention measure of TABL (e.g. closely monitor animals with a radiance >1x107 photons/s for LL/2-Luc) is derived during the model development process at a point prior to the onset of clinical signs, which is a refinement that minimizes further harm to the mice.

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

Mice are the lowest species in which the knock out of the immune system allows growth of human tumours. Mice with a fully functioning immune system also provide the opportunity to investigate the immune system interplay with a mouse tumour.

The architecture of mammals is required to accurately model tumour development and spread to relevant organs. Work on human tissue and use of mixed tumour-derived cell

types, especially when freshly derived from a patient should allow the most refined assessment of novel anti-cancer agents. Transgenic technology and can also be used to assess the importance of potential oncogenes and mice are the lowest species in which this technology can be applied to as this requires an appropriate mammalian architecture.

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

The development of relevant pre-clinical models of oncology in Objective 1 is a key stage for the evaluation of candidate anti-cancer agents to ensure the right models are being used to answer the questions being addressed in Objective 2. The following will be undertaken to minimize animal suffering across all models;

- Pilot studies for the establishment of new tumour cell lines and refinements to surgical techniques will be carried out on an ongoing basis and the advice of the NVS and/or the NACWO will be sought.

- Use of pilot tolerability studies to ensure there are no unexpected adverse effects associated with new models and/or unexpected toxicity because of tumour:drug interactions, and to ensure the drug levels used are not associated with any cumulative effects

- All surgical procedures will be conducted in line with established welfare guidelines on aseptic surgery, using suitable anaesthesia along with peri-operative analgesia. Where there is a scientifically justifiable reason for concern that the mechanism of action of an analgesic regimen may affect cell growth and tumour initiation, advice will be sought from the NVS as to suitable alternative forms of analgesia i.e. those with a different mechanism of action.

- When using non-labelled cell lines/tumour fragments (e.g PDX material) the frequency of animal monitoring will be increased when there is evidence of nearing potential study endpoint e.g. observation of body weight loss and/or other potential indicative clinical signs.

- All studies will be conducted in accordance with established welfare guidelines. Presentation of adverse clinical signs, behaviour patterns or body weight loss relating to treatment or model progression should be de-risked by supporting work summarised above, and managed as detailed in the relevant project plan and protocol sections that will accompany each individual study.

- Animal husbandry and enrichment will be constantly reviewed and updated according to best practices. Cardboard tubes and housing will be used where possible to aid with wound healing following any surgical intervention.

- Animals that are prone to hind limb issues, e.g mice bred on a SCID background, will be given soft bedding and non-adverse handled. Food may also be placed directly into the cage to prevent animals having to reach up onto their hind limbs in order to access food hoppers.

- Any in-life sampling or terminal sampling will be in line with established welfare guidelines and micro-sampling regimens will be utilised where study design supports this.

- The frequency of dosing will be such that animals fully recover between dosing occasion and will not suffer more than transient pain and distress and no lasting harm and there will be no cumulative effect from repeated dosing.

- For models that have a defined organ of tumour establishment (e.g. pancreas for pancreatic models, bladder for bladder models), evidence of tumours seeding within other organs/sites, without evidence of a primary tumour within the intended organ, will result in animals being humanely killed.

- The use of supplemented diet or drinking water may be used for both candidate anti-cancer agents as well as hormone supplementation, but in such circumstances, care should be taken to carefully monitor intake to ensure that that the change in composition doesn't affect normal feeding/drinking behaviour. For hormone dependent models (some oestrogen-dependent breast/ovarian models) hormone supplementation using the most refined method that results in consistent tumour growth for efficacy testing will be utilised (namely supplementation via the drinking water, hormone rods, pellets, or mini-pump devices). Alternative supplementation in the diet and/or water has been tested and further refinements will be evaluated.

- If the animals have oestrogen supplementation then there may be evidence of the following: Bladder calculi may lead to urinary retention resulting in an enlarged bladder with abdominal distension. We may use ultrasound to monitor the bladder calculi where appropriate. Animals may also experience urine scald. Any animal exhibiting these signs, or evidence of a grade 3 urine scald (reddened, broken skin with possible discolouration and wet around the genital area),or a deterioration in clinical condition will be humanely killed. Oestrogen supplementation via drinking water has, in our hands, resulted in growth of an oestrogen-sensitive cell line and urine scald and bladder calculi were not observed. Where possible we will look to validate our models using oestrogen supplementation via the drinking water, however this may not be possible for cell lines that need high oestrogen concentrations. We have also found that using certain strains of mice e.g. NSG avoids the occurrence of bladder calculi and urine scald following oestrogen supplementation, and where possible, this strain of mouse will be used in new model development or re-validation studies.

- Where a statistically significant effect on study goals can be determined prior to the end of the scheduled dosing phase, the study will be terminated at that point.

- Through continual professional development, our company seeks to improve and implement new techniques for current/new models, which are developed through dialogue and guidance from the NVS or alternative veterinary expertise, to ensure that the most refined methods are considered.

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

Surgical procedures will be carried out in accordance with the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery.

For administering substances the Laboratory Animal Science Association Good Practice Guidelines for administration of substances will be followed.

Frequency and volume of blood collection will be carried out in accordance with the following guidance https://www.nc3rs.org.uk/3rs-resources/blood-sampling)

Planning and reporting of experiments will be in accordance with Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) and Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

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

The following practices are already in place:

- Regular interaction with the National Centre for the Replacement, Refinement and Reduction in Animals in Research website (https://www.nc3rs.org.uk) and subscription to regular newsletters.

- Regular discussions with named role holders (i.e NIO, NVS, NACWO & NTCO) and in-vivo colleagues (i.e animal scientists, technicians and care staff).

- Attending appropriate training courses and conferences or seeking feedback from colleagues attending these events.

- Liaising with AWERB members.

We have an AWERB committee that regularly meets. Members of the AWERB committee include the PPLh, NTCO, NACWO, NIO, PEL, NVS and a lay person. During the meetings, internal R&D (Research and Development) data is presented and discussions around future R&D take place. During this meeting advice is given on latest and best practices to include on the R&D studies. Following completion of the model development phase, a power point summary of the data will be generated and the outcomes of the model development process will be carefully reviewed by the AWERB/R&D committee before the model is considered suitable for use in client studies. As part of the ongoing commitment to the highest levels of scientific and welfare, a regular review period will be set up for each model following completion of the model development process. This will look to follow-up on the current use and applications of the model to ensure that the most refined science and animal welfare is being utilised. Where areas of potential refinement are identified, these will be assessed as part of further pilot studies.

11. Mechanisms of Computation in the Brain

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Neural Circuits, Computation, Brain mechanisms, Information processing in the brain, Neurological disease

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

Our research aims to understand how the brain processes information and how this affects behaviour. To do this, we will study how networks of brain cells, neurons, work together to analyse sensory information and produce behavioural responses. We will also investigate the connections between neurons and how they influence the processing of information.

This will contribute to elucidating the mechanisms of how the mammalian brain computes, what the algorithms are and how are they implemented. It will create fundamental knowledge and a basic understanding of brain function. This is not only critical for furthering our understanding of those brain mechanisms perturbed in psychiatric and neurological diseases such as autism or schizophrenia but a fundamental quest of human curiosity.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 will be using small mammalian neural networks for our research, the brain circuits of mice and rats. This will allow us to study the intricate interplay between different neurons in detail, as well as the identity and connectivity of excitatory and inhibitory elements of the network. By doing so, we can gain a more comprehensive understanding of how neural networks process information.

Our research also has important implications for understanding cognitive disorders such as autism and schizophrenia. These disorders are linked to changes in neural network structure and function. By studying small, compact neural circuits in mammals, and using computational models, we hope to gain a better understanding of the causal and mechanistic links between neural networks and behaviour.

This will provide a solid foundation for assessing the impact of cognitive disorders on small neural networks.

In summary, our research aims to understand how networks of neurons process and analyse sensory information and produce behavioural responses. By studying the interactions between different brain cells and their components in small neural networks, we can gain insight into the fundamental processes that underlie brain function. Our research also has important implications for understanding cognitive disorders and assessing their impact on small neural networks.

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

This project will generate new scientific knowledge about how information is represented and processed in the brain. We will publish our results through publications in high-quality peer-reviewed scientific journals. We will make datasets on e.g. neuronal recordings or neural structure available in an accessible, browsable, curated, and reusable way. These will allow other researchers to dig deeper into analysis of existing datasets, thereby maximising knowledge that can be derived from our work.

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

This is a basic research program that provides fundamental insight into how information is processed in small neuronal networks. Direct translational relevance, however, can come from technological developments that improve the way we can record electrical activity from brain cells. This in turn might be applicable to human brain-machine interfaces, which are discussed as key tools to help physically disabled patients reconnect with the external world.

Information processing in small neural networks is a key area where our understanding is still very limited, yet most complex neurological diseases such as autism spectrum disorders and schizophrenia are believed to be based on alterations not on the cellular or regional level but exactly on the level of small networks. We will specifically gain novel information as to how sensory information is processed. We hope to discover novel computational algorithms which can describe how such processing is implemented in small neural networks and advance our knowledge of sensory systems and their performance in general. This together will set the basis for an understanding of brain function in disease. Thus, while in essence primarily advancing fundamental scientific understanding, our results may be relevant for future studies aiming to treat human disorders.

We will aim to present and disseminate our work at national and international conferences and disseminate the gained knowledge and understanding through publishing our work in high-impact peer reviewed journals and early dissemination through PrePrint servers (BioRXiv). The new tools developed in this project will be valuable to other scientists investigating sensory information processing. Especially the quantitative behavioural approaches will help others to sensitively study e.g., the impact of drug treatments on behaviour. We will make such technology, as well as animals, protocols, insights and reagents available to other researchers on e.g., AddGene, JAX, GitHub, FigShare or other open access databases and resource hubs. We will furthermore assess on a case- by-case basis whether dissemination through companies would be an additional route to rapidly distribute technical developments to assist other researchers in their research. Moreover, presenting the work to broad audiences has the potential to foster relationships with or build new commercial entities in the healthcare space (e.g. spin-outs that develop implantable neural devices to help locked- in and paralysed patients).

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

During this project, we will actively collaborate with colleagues not only from neuroscience, engineering, and mathematics backgrounds. We will in particular collaborate with neurologists and neurosurgeons to explore where systems neuroscience methodology (experimental and analytical) can directly aid neurosurgical procedures and neurological diagnosis. Moreover, we will seek collaborations to theoretical neuroscientists and machine learning experts to assess where algorithmic properties uncovered in brain circuits can impact on the development of machine learning tools.

Finally, and most generally, we will maximise outputs through the aforementioned rapid publication of results on preprint servers (BioRXiv) as well as through publishing raw data on suitable platforms (figshare, WebKnossos), algorithms and protocols on appropriate outlets such as GitHub and negative results on Wellcome Open Research.

Species and numbers of animals expected to be used

- Mice: 8400
- Rats: 20

Predicted harms

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

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

Mice are the species with the lowest sentience that are still suited for studying mammalian brain function. Therefore, mice have been extensively used in neurophysiological and systems neuroscience research. As a result, all the necessary assays and reagents for this project are available for mice, as well as extensive atlas knowledge, reducing the number of animals needed. As we aim to unravel algorithms and computations in their mature form, most experiments focus on adult mice. In some cases, the emergence of computation will be particularly informative, necessitating some experiments at earlier developmental stages. Thus, we will work predominantly with adult mice and in these rare cases with juvenile mice.

When attempting to record brain activity with tools that are designed to obtain information from human brains, thereby e.g., guiding clinical decisions, the geometric dimensions of these probes often preclude employing them in mice due to the small size of their brain . Thus, in these cases we will resort to adult rats that have a similar neuronal architecture yet make it possible to use human-size probes in their substantial larger brains.

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

Animals will undergo behavioural experiments. During these experiments, animals will be presented with stimuli such as smells, lights and sounds. All the stimuli are below the pain threshold, and animals will be habituated to them over the course of days. In response to these stimuli, animals will typically poke their snouts into openings and receive water as a reward. In some behavioural experiments, animals will be unable to move their heads as they will be mechanically restrained. This is necessary to allow for measurements of brain function that require a still head. To make sure that animals are motivated to perform the experiments, water may be restricted outside the experiments. However, we will make sure that animals maintain good health and weight by supplementing them with additional amounts of water outside the experiment or by providing them with free access to citric acid water.

Such citric acid water is not as palatable but equally hydrating as normal water.

Some animals will undergo surgical procedures. A portion of the experiments will involve the insertion or implantation of a device such as a silicon recording probe, glass fibre, recording window, or cannula into the brain. Most of these approaches will cause little to no discomfort or pain as the brain itself does not feel pain (e.g. patients in neurosurgical settings are often awake). Some experiments will involve functional imaging from the brain where the brain surface is illuminated or scanned with a light source to measure activity in the brain. Some experiments will also involve the injection of a gene-delivery agent (such as a virus used as a gene ferry) that allows to label specific cells. These labels will allow us to monitor activity from cells or even control activity (e.g. exciting or inhibiting cells to test predictions of computational models). To alleviate pain and prevent infections, the procedures will be conducted under anaesthesia and aseptic conditions. Postoperative pain and inflammation will be closely monitored, and animals will receive preventive pain killers during and after the surgery. Animals will be left one week to recover before undergoing any other procedures.

Experiments typically run for 1-12 months (rarely 18), where animals perform e.g. repeated behavioural experiments or different sessions of functional recordings.

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

During the behavioural experiment, the animals might experience stress which we plan to minimise through gradually habituating animals to the new environments. Animals typically show signs of distress in the form of increased activity for around five to fifteen minutes after entering the experimental environment for the first two to three times until they get used to this situation. Water restriction outside the experiments might lead to thirst and weight loss, which we will minimize by ensuring e.g. a minimum water intake.

Animals may experience pain during injection of substances which typically lasts for minutes.

Animals will experience some pain and distress after surgery, which we aim to minimise with pain killers and careful monitoring, and which typically resolve after a few 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)?

For mice:
Mild - 50%
Moderate - 50%
Severe - 0% For rats:
Mild - 100%

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

- Killed
- Used in other projects
- Kept alive

Replacement

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

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

In this project we aim to understand how neuronal circuits process information through the interplay of specific neuronal connections, the excitability and integrative properties of individual neurons and the arrangement of sensory inputs. While a lot of information and insight about the properties of individual neurons and anatomy has been acquired over the last decades, it is still essential to uncover the fundamental principles in live tissue. These experiments can indeed be guided more and more specifically through detailed simulations and computer models (as we are routinely doing) but it is both critical to fill the gaps in knowledge of model parameters, as well as test the predictions of such models experimentally in order to uncover the workings of the brain in health and ultimately understand the specific deficits in disease. As we learn more about neurons and synapses under investigation, we will be able to use mathematical modelling more extensively, but for such approaches to be useful, they will need to be tightly constrained by biological measurements.

For this project we will extensively rely on insight generated from in depth analysis of existing data, especially anatomy and single cell physiology. Furthermore, we will identify gaps in our anatomical knowledge and perform ex vivo analysis of neural circuits using electron microscopy. Similarly, where cellular properties are not sufficiently known to constrain models, we will generate detailed biophysical models of individual cells and perform necessary in vitro experiments to obtain such data. We will put this data together in computer models in order to generate specific hypotheses and guide our experimental efforts.

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

Following the PREPARE guidelines and RSPCA suggestions, we considered cell cultures, simpler non- vertebrate organisms, mathematical and computer simulations, and studies in human volunteers. We routinely explore this by searching biomedical databases such as PubMed, Google Scholar, Web of Science. Additionally, we consider and attend conferences in particular computational conferences such as COSYNE and NeurIPS as well as by engaging with non-vertebrate neuroscience. Finally, we are increasingly exploring how to employ invasive electrophysiological recordings in patients undergoing clinical procedures in order to complement animal experiments.

Why were they not suitable?

We will supplement our research with the identified non-animal alternatives to refine our hypotheses whenever possible, but our objectives cannot be achieved by non-animal alternatives alone. Isolated cell cultures are not able to describe computations underlying behaviour. Similarly, simpler non- vertebrate organisms are not suited to reproduce the complex behavioural and computational abilities displayed by mammalian brains. Mathematical and computer simulations are unable to recapitulate the unknown biological algorithms let alone biological implementation – which is in turn critical to unravel those mechanisms that are ultimately perturbed in neurological disease. There is limited opportunity to investigate the function of individual neurons in humans. While we actively explore these alternatives with neurosurgeons, mechanistic investigations in humans are intrinsically limited by accessibility and the lack of opportunities to investigate causal links between neural circuit structure and brain function. The available methods for studying brain function non-invasively (functional imaging, electroencephalography, transcranial magnetic stimulation) lack the temporal, spatial and biological resolution required to achieve our objectives.

Reduction

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

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

Where physiological or other interventions are required, we expect that 5-6 animals per treatment group will usually be sufficient to obtain robust results. For most of the quantitative experiments, design will be based on PREPARE guidelines and sample sizes may be set using power analysis, generally using a significance level of 5%, a power of 80%, and a least practicable difference between groups of 20%. Otherwise, we will use the minimum number of animals to provide an adequate description, generally on the basis of previous experience (our own and from the literature). We have statistical expertise within our group and at the Institute to adequately determine sample size based on power analysis. We will use ARRIVE guidelines when we publish our work to assure reproducibility of our research.

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

We have spent considerable effort in developing technology for large-scale neural recording that will allow recording as much data in a single animal experiment as previously was obtained with dozens of animals, thus substantially reducing the number of animals being used. Additionally, we have extended our anatomical efforts, combining high resolution volume electron microscopy with synchrotron computed tomography and automated data analysis tools. This allows us to obtain connectome information from a single experiment rather than combining a large number of e.g., viral tracing experiments, again drastically reducing the number of animals needed. Finally, for behavioural experiments we now employ socially housed, home cage behaviours that allow us to work with a healthy cohort of mice over period of up to 18 months, obtaining data that with traditional methods would have required many individual cohorts of mice.

All three allow us now to obtain vast amounts of data in individual experiments, thereby substantially reducing the number of animals needed compared to e.g., whole-cell patch or tetrode recordings or viral tracing respectively (by at least 2 orders of magnitude). Furthermore, to complement our statistical expertise gained during more than 20 years of active research, we will consult with biostatisticians experiments in particular, we will mainly use repeated-measures experimental designs, which will reduce the number of animals needed as compared to standard grouped designs.

In general, lines will be maintained in a homozygous state, to limit the number of offspring with genotypes that cannot be used in the experimental settings. If it is unavoidable to use heterozygote breedings to generate homozygotes, littermates genotyped as heterozygous or wild type will be used as age and gender matched controls.

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

To achieve our objectives, we will need transgenic mouse lines. In collaboration with the local biological research facilities, we will develop and maintain the most efficient breeding, minimising the number of animals during breeding. This will include breeding from homozygous breeders to ensure that all offspring have a suitable genotype as well as cryopreservation of embryos to enable breeding only when animals are needed. To further minimise the number of animals, we plan to minimise individual variability by using in-bred strains with genetically homogenous backgrounds. To further reduce animal numbers, animals will be efficiently used whenever possible without adverse effects on animal welfare. For instance, when animals have successfully undergone non-invasive behavioural training in a task, they may be transferred to another protocol to test the effects of different experimental interventions on behaviour. This will reduce the number of animals required as compared to the alternative of establishing a behavioural task by training one cohort of animals and testing the effects of experimental interventions by training and testing a new cohort of animals. Moreover, we will minimise the number of animals by maximising the amount of data gained from one animal whenever possible as described above. All experiments will be conducted in animals of both sexes, unless a scientific reason suggests the use of one sex.

Refinement

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

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

Which animal models and methods 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 for this project that are sufficiently close to humans to reveal principles of information processing in the brain. Furthermore, rodents have been a long-standing useful model for behavioural studies of learning and memory, which enables us to build upon a large body of research already carried out, and to relate our findings to previous results. Experiments will be performed in wild-type animals (around 60%) and genetically altered animals (40%).

For behavioural analysis we have developed a group housing approach that ensures rich social interactions, free access to food and ad lib access to water, thus reducing experimenter handling and other potential stressors. Additional experimental approaches include stimulation and recording from a slice preparation ex vivo, and from different regions of mammalian brain in vivo. These are state-of-the-art refined tools that can be used for the study of information processing and connectivity in genetically and anatomically defined neural networks.

We will do all surgical procedures, especially stereotaxic injections in the brain and implantation surgeries under strictly aseptic conditions to prevent any post-surgery infections. Post-surgery animals' recovery is directly monitored in a recovery chamber until the animal is returned to its home cage.

Typically, once animals are returned to their home cage they are observed at least twice daily. Experiments will be done in awake animals where our previous data and the data of colleagues would indicate that anaesthetised animals might significantly deviate from information processing in the awake, healthy brain. Furthermore, we will typically group-house mice and provide environmental enrichment, which reduces stress levels significantly compared to isolated housing.

With the help of the biological research facility, in particular the named information officer and the vets, we constantly monitor best practices and new information available in the international literature and on the NC3RS and RSPCA websites (www.nc3rs.org.uk/our-resources, www.rspca.org.uk/adviceandwelfare/laboratory).

We plan to use a small (<20) number of rats in cases where neural recording procedures are used with tools that mirror those used in human patients. In these cases, the larger brain of the rat allows using identical tools to the clinical setting, thereby collecting data in animal experiments that is directly comparable to the clinical setting.

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

This project aims to unravel how information is represented and processed in the mammalian brain. We strive to unravel mechanisms that allow us to further our understanding of how the healthy brain computes – which is essential for our understanding and treatment of complex neurological diseases such as schizophrenia or autism. While “lower” species provide very interesting model systems for fundamental aspects of computation (and inform the experiments we and others perform on mice), the

nature of e.g., insect brains is too dissimilar from mammalian brains (in size, complexity, composition, wiring, as well as in the properties of individual components) to allow direct conclusions for the human case. For the work to be translatable to human patients, mammalian species are needed. Mice are well established in neuroscience, provide key tools such as genetic access and are to the best of our knowledge the species with the "lowest sentience" still replicating key aspects of the human brain.

Transgenic animals offer access to specific cell populations as well as providing certain disease models. For recording and perturbation access (key for causal studies) to specific cell populations, viral gene delivery is crucial as this allows precise spatiotemporal access. As viruses need at least several days (typically several weeks) for stable expression, surgical procedures under anaesthesia have to be followed by recovery time and subsequent e.g., neural recording procedures. Moreover, from our own and other laboratories' work it has become obvious that computation in the brain is substantially influenced by anaesthesia. Therefore, recordings need to be performed in awake animals.

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

To reduce the stress associated with behavioural experiments, all the delivered stimuli are below the pain threshold, and animals will be frequently handled and habituated to the experimental setup over the course of days. To minimise thirst caused by the water restriction, we will either ensure that animals receive a minimum of 40mL/kg water (roughly equivalent to 1/3 of their body weight) or we will provide animals unrestricted access to citric acid water (less palatable but equally hydrating as regular water – in which case intake will be closely monitored to ensure sufficient intake). Furthermore, wherever possible we provide appetitive rewards such as sweetened or soy milk rather than water, reducing the need for water restriction. Whenever possible, neural recordings will be carried out in freely moving animals using lightweight implants that are easily supported by the animal, but, in some cases, head- restraint may be needed to enable valid results. In this case, animals will be habituated to the recording setup in incremental steps starting with short durations on the order of several minutes.

Where possible, we will perform behavioural experiments in a social housing setting, that we have developed and that has received widespread praise for its refinement (e.g., 3Rs prizes, dissemination with support of the NC3Rs etc). Here, continuous access to water and self-initiating of trials ensures a self-guided hydration state and reducing potential discomfort or distress due to water restriction.

Surgical procedures will be conducted under anaesthesia and aseptic conditions to alleviate pain and reduce the risk of postoperative infection. Postoperative pain and inflammation will be closely monitored, typically twice a day. Animals will receive preventive pain killers during and after the surgery. Animals will be left one week to recover before undergoing behavioural experiments.

Animals will be observed every day by a person experienced in animal husbandry to identify potential adverse events and ensure that humane endpoints are adhered to. We will typically group-house animals and provide enrichment including nesting material to increase animal welfare.

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

Whenever applicable, we will follow the best practice guidelines provided by the NC3Rs (e.g., for blood draws or for husbandry). For surgical and non-surgical procedures, we will follow the recommendations of the Laboratory Animal Science Association (https://www.lasa.co.uk/wp- content/uploads/2018/05/Aseptic-Surgery.pdf) and of the Procedures with Care website (https://researchanimaltraining.com/article-categories/procedures-with-care/) as well as consider and implement best practice as outlined in community- and NC3R-led systematic reviews such as Barkus et al, J Neurosci Methods (2022) 381:109705 "Refinements to rodent head fixation and fluid/food control for neuroscience".

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

We constantly monitor best practices and new information available in the international literature and on the NC3RS and RSPCA websites (www.nc3rs.org.uk/our-resources www.rspca.org.uk/adviceandwelfare/laboratory). I am also subscribed to the newsletter of the NC3RS (www.nc3rs.org.uk) and follow the RSPCA twitter account dedicated at laboratory animal welfare (@RSPCA_LabAnimal). Moreover, I am the local liaison with our local N3R regional programme manager and regularly consult with her. Finally, we have actively participated in several NC3R working groups and make use of this extensive network as well as meetings, workshops and conferences to continuously adopt best practise e.g., in surgical workflows.

12. Advancing the knowledge of mRNA 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

RNA, Peptides, Chemical modifications, Nucleic acid delivery, Nanoparticles

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

mRNA therapy is an innovative approach to treating diseases that utilises messenger RNA (mRNA) as a therapeutic agent. mRNA is the genetic material that serves as the blueprint for producing proteins in the body. In mRNA therapy, the mRNA is engineered to encode a specific therapeutic protein and delivered into the patient's cells, where it directs the production of that protein. This process allows the body to produce its own therapeutic proteins, which can then treat the underlying disease.

Peptide therapy, on the other hand, is the use of lab-made peptides to trigger a specific reaction in the body. Peptide therapy aims to raise low peptide levels that can't be replaced with supplements to help prevent or treat certain conditions.

Our goal is to combine the potential of these two therapeutic agents by developing mRNA to express therapeutic peptides. However, poor cellular uptake and the stability of RNA molecules are major impediments to clinical applications.
The primary objectives of this project are to (i) identify stable mRNA molecules that can be efficiently translated in cells over time, and (ii) develop novel delivery methods for mRNA to

the liver, where it can be translated to a peptide which will be secreted into the bloodstream.

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

Why is it important to undertake this work?

mRNA therapy represents a promising direction in medicine, offering a safe and effective way to treat a wide range of diseases without the need for permanent genetic modification. With its rapid and easy manufacturing process, potential for personalisation, and positive early clinical trial results, mRNA therapy has the potential to revolutionise disease treatment. One promising application of mRNA therapy is the expression of secreted therapeutic peptides.

Peptide-based therapy has been successfully applied to treat metabolic diseases, allergic diseases, infectious diseases, autoimmune diseases, fibrosis, and asthma. Therapeutic peptides offer several advantages over proteins or antibodies: they are small, easy to synthesise, and can easily penetrate cell membranes. Additionally, they have high activity, specificity, and affinity; minimal drug-drug interaction; and biological and chemical diversity.

Despite its potential benefits, there are several challenges that must be addressed to fully unlock the potential of mRNA as a therapeutic option. Some of the main challenges include:

Delivery: One of the main challenges in mRNA therapy is safely and effectively delivering the mRNA to the appropriate cells. The mRNA must enter the cells and reach the ribosomes, which are responsible for protein synthesis.

Stability: mRNA is a fragile molecule that can be degraded by cellular enzymes. Researchers are exploring ways to modify the mRNA molecule to increase its stability and improve its ability to enter cells.

Immune response: The immune system may recognise the introduced mRNA as foreign and mount an immune response against it, potentially reducing its efficacy. Researchers are exploring ways to reduce this immune response, such as modifying the mRNA to avoid triggering an immune response or co-administering immunosuppressive drugs.

In contrast to long mRNAs, which are currently synthesised by enzymes such as the bacteriophage T7 expression system, mRNA encoding short peptides can be chemically synthesised. This unlocks the advantage of synthesising modified mRNA encoding these peptides. We have developed a unique chemistry that allows us to screen for a wide range of modifications, which increases mRNA activity and stability.
This program aims to develop new mRNA sequences and identify chemical modifications that will enable the stable expression of secreted peptides. Additionally, the program will develop a solution for delivering mRNA to the depot organs (such as the liver), which naturally synthesises and secretes various proteins and hormones into the bloodstream. Once the mRNA is translated in the depot organ, the peptide will be secreted to the

bloodstream and, from there, reach the target organ.

Importantly, the results of this project will be highly relevant for any nucleic acid-based therapy.

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

Peptide-based therapeutics have huge potential for treating a range of diseases, and a number of peptides are already approved or under investigation for the potential treatment of metabolic diseases, viral infections, and more.

Using long-acting mRNA instead of traditional peptide-based therapy has the potential to significantly improve patients' lives, as it requires less frequent administration.

However, nucleic acids are susceptible to degradation, have immunogenicity, and the complex structure of the target organs can retard the effective delivery of nucleic acid drugs. Therefore, the clinical application of nucleic acid therapy ultimately relies on well-developed carriers and methods to ensure safety and efficacy.

The research outlined in this proposal aims to make progress in the area of nucleic acid therapy. We aim to identify (i) specific mRNA sequences that can be used to express secreted therapeutic peptides,
(ii) specific modifications that will increase mRNA potency, and (iii) methods that can efficiently and safely deliver mRNA to the target depot organ.

We have developed and tested multiple therapeutic mRNAs in vitro that can be used for the secretion of therapeutic peptides. The results from this project will be integrated into these studies to create safe and effective treatments for specific diseases.

Publications resulting from this project and all mouse models generated will be made available to the scientific community and could be important for the scientific advancement of other researchers.

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

In the short term, the project outputs will provide new information that benefits the scientific and translational communities through collaborations and publication of our work. This information will fill gaps in current knowledge and serve as a basis for further investigations into the biology of nucleic acid therapy.

Nucleic acid delivery is the main barrier, holding back the field of nucleic acid therapy from moving faster to the clinic. The delivery systems and protocols which will be developed as part of this study will have significant contributions to the field of nucleic acid-based therapeutics. Moreover, it will provide guidance for the delivery of nucleic acids to other organs and tissues. In the mid-term, our research strategy will enable a faster translation of mRNA therapy from the bench to human clinical trials. Our project will provide significant evidence regarding the efficacy and safety of the mRNA and delivery vehicles in mice and, as a result, increase confidence in their success in human subjects. Benefits at this stage will be seen by the translational research scientific community and clinical scientists.

Our long-term goal for this project is to enable the successful use of mRNAs in clinical practice for treating a range of diseases. Although our focus is on developing efficient, long-lasting, and safe mRNA delivery methods, we are already testing the efficacy of our mRNAs in relevant rodent and non- rodent disease models in collaboration with academic institutes and CROs. All of these studies are designed to fit the standards specified in this PPL to ensure a smooth transition. Having these studies on our R&D site will allow us to (i) execute studies which require fresh material, such as single-cell sequencing, (ii) use instruments such as the IVIS, which are not available in all CROs, and (iii) have better control over the execution of the studies. This includes, for example, the ability to randomise our study and have staff members who are specifically trained in the techniques required for these studies. We will keep working with CROs when using animal models not covered by this PPL (such as NHPs) or for specific disease models.

The combination of the external and internal will allow us to bring safe and long-lasting mRNA therapy to the clinic.

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

To maximise the output of our work, we are collaborating with multiple companies and academic labs. For the development of nanoparticle-based delivery, we are collaborating with establishments in Europe and the US. For the development of mouse models to monitor mRNA delivery to specific organs, we are collaborating with additional CROs in the US and Europe, as well as an academic institution in the US. Similarly, we are collaborating with other companies and research labs to develop a high-throughput method for identifying chemical modifications that increase mRNA translation and stability. We regularly share information from this project with our partners to support their research.

We aim to maximise the output of our work through open communication and sharing of our progress with academics, clinical professionals, and the general public. We will use a variety of platforms, such as conferences, publications, and our website. All scientific results will be published in open-access journals in a timely manner, as per company policy, to ensure that the information presented is widely available.

Species and numbers of animals expected to be used

- Mice: 5500

Predicted harms

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

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

This project aims to (i) develop safe, effective, and long-lasting methods of delivering nucleic acids, and (ii) test their activity in inbred wild-type mice and disease models. To fully understand the activity, durability, and safety of nucleic acids in the depot organ, an in vivo model is necessary to encapsulate the target tissue's complexity, cellular interactions, and microenvironment. Moreover, in vivo models are the only system that can evaluate the secretion of the peptide from the depot organ to the bloodstream and its delivery to the

effector organ. Finally, the use of available rodent disease models is crucial for evaluating the efficacy of treatment.

We are using in vitro models, including primary cells and organoids, to test our mRNA molecules and peptide secretion. However, currently, no models can mimic the systemic vasculature or interaction between circulation and the depot organ in vitro or in silico. Live animals and tissue are necessary to test the delivery of mRNA to the depot organ from the bloodstream, mRNA stability within the organ, protein secretion from it, and the peptide recipient in the effector organ. Moreover, these are the only systems that can predict mRNA biodistribution in other organs and its effect on the immune system. While using cell lines and other in vitro assays reduces our use of animals, it cannot answer all questions that can be addressed by in vivo models where the biological systems are similar to those of humans.

Rodent models are the most widely used in vivo models in research. Among rodents, mice are considered to be the least sentient species that still demonstrate pharmacokinetic and pharmacodynamic characteristics similar to humans, given that we share over 95% of the same genetic background (source: https://www.jax.org/genetics-and-healthcare/genetics-and-genomics/why- mouse-genetics).

The scientific community has developed a range of techniques that enable manipulation of the mouse genome, allowing us to generate models to answer specific key questions. The models described in this project are optimal for studying nucleic acid activity and durability while minimizing the number of animals used and reducing their suffering.

The studies outlined in this project will involve the use of adult mice in which all organs are fully developed.

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

This project will use traditional inbred wild-type strains, such as Balb/c and C57bl6, as well as genetically modified mice. Genetically engineered mouse models (GEMMs) will be used to test the effect of mRNA treatment on specific disease models, such as metabolic diseases.

Breeding of genetically modified animals will be done through natural breeding behaviour. These mice have been genetically modified to overexpress a disease-causing gene or have a gene deletion. When CRE (Carbapenem-resistant Enterobacteriaceae; bacterial strains which are resistant to some antibiotics) expression is required, a gene inducer (e.g., tamoxifen) will be administered through drinking water, food, or injection. This may result in the birth of animals that will spontaneously develop a specific disorder as they age due to the activation of the disease-causing gene or gene deletion.

Some animals will receive a specific diet to induce metabolic disorders. Diet-induced metabolic syndrome has been widely used in studies of obesity and other metabolic disorders. This can be achieved by administering a single type of diet, such as high-fructose, high-sucrose, high-fat, or a combination of those.

The animals will be monitored for metabolic disorders by tracking their weight or metabolite levels in their urine and blood.

Nucleic acids, with or without a delivery vehicle or the therapeutic peptide, will be administered using one of the following well-established methods:

Intravenous (IV)

Intraperitoneal (IP)

Subcutaneous (SC)

The animals will be monitored for peptide levels in their blood and for changes in the disease phenotype by measuring their weight, behaviour, and metabolite levels in their urine and blood. This monitoring will continue until the protein and metabolite levels in the blood return to their baseline levels before treatment.

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

Many of the animals used in this project are not expected to experience suffering. Our mRNA molecule will express endogenous peptides, which have already been tested as peptide treatment. Our mRNA therapy will result in the expression of the same peptide sequence. Therefore, we do not anticipate any adverse effects resulting from their expression or secretion into the bloodstream.

Inducing metabolic diseases will affect the animals' weight, and they are expected to gain 20-30% of their initial body weight. The animals may develop health conditions, such as insulin resistance, glucose intolerance, hyperlipidemia, and fatty liver disease, as well as inflammation, cardiovascular complications, and changes in behaviour, such as food intake, decreased physical activity, and changes in social interaction patterns.
External mRNA delivery can activate innate immune responses, including the production of type I interferons and pro-inflammatory cytokines. However, mRNA and nanoparticle delivery to the liver are well tolerated in animals and humans without significant liver toxicity. Nevertheless, repeat dosing may cause liver toxicity or inflammation.

All animals will be closely monitored for these effects during the study.

Expected severity categories and the 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 15% of the animals used in this project will experience a moderate level of severity, while the remaining 85% will be expected to fall into the mild or subthreshold category.

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

- Killed

Replacement

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

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

At this stage, achieving our goals of developing nucleic acid therapy requires testing the efficacy of our mRNA molecules and delivery methods in vivo to assess the efficacy and stability of the nucleic acid in the liver.

Moreover, animals are required to achieve our aim as regulatory and research bodies require a preclinical assessment of potential therapies in animal models before their translation to the human clinic.

Despite this, we will continue to use tissue culture methods and machine learning tools to optimise the selection of RNA molecules and their chemical modifications, as well as the performance of our delivery vehicle. This will enable us to improve the selection of RNA molecules, production of the delivery vehicle, and complexation procedure. By taking these steps, we can minimise the number of animals used in testing to only those that have shown the most promising effects.

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

In the careful design of a research project, we considered methods that would minimise the use of animals, such as primary hepatocytes, hepatocyte spheroids, and liver organoids. However, these systems do not always fully model the complex nature of the liver or protein secretion from the liver to the bloodstream.

When possible, we will use alternative research approaches that do not involve animal testing. In particular, we will use in vitro methods with primary cells from healthy and patient donors, or liver organoids, to screen for the efficacy of RNA molecules and their delivery.

Why were they not suitable?

While the use of cell culture methods can help reduce the use of animals in research, they cannot completely replace them since they do not capture the full complexity of organs, including protein secretion into the bloodstream and the effects of these proteins on the receiving organ. Organoids, cell cultures, and primary cells also lack many relevant features, such as the delivery of nanoparticles and mRNA molecules from the blood to the depot organ, the immune response to mRNA or peptides, and the physiological effects of peptides in disease conditions. To develop efficient and safe delivery to depot organs, models that encapsulate its complexity and microenvironment are necessary. Only such models can enable testing of the dynamic action and long-term effects of RNA molecules in a specific organ. Additionally, regulatory bodies require preclinical assessment of any new modality prior to human trials. Therefore, it is critical to use the best models and methods in this research.

Reduction

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

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

Each of our mouse model experiments has a careful statistical design that is aimed at minimising the use of animals while ensuring robust and meaningful statistical endpoints. These animal numbers are selected in collaboration with highly qualified statisticians and are based on our experience from the last years with mRNA delivery to mouse models (including work with CROs and work on PPL (P9762231)).

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

Our project aims to reduce, replace, and refine the use of animal models as much as possible in all designed experiments. To achieve this goal, we will use cell culture methods to replace or reduce the number of animals used in studies whenever possible. For instance, in trials of new sequences or chemical modifications, we will first determine the efficacy and safety of these molecules in primary hepatocytes, cell lines generated from animal models, and 3D liver organoids. Similarly, new batches of our delivery vehicle will be tested in vitro for safety and delivery efficiency. We will use stringent downstream analysis methods for these tests. The results will indicate whether a mouse study should be conducted and how to conduct it.

When mouse model experiments are necessary, we have consulted experienced in vivo statisticians to design our studies in a way that minimises animal use while achieving reliable statistical endpoints.
These calculations take into account the fact that (i) mRNA delivery to the depot organ, its translation, and protein secretion vary between animals, and (ii) the physiological effect in disease models varies between animals. We will continuously update the number of animals required for each study based on accumulating data.

In many animal studies, we have included a bioluminescent reporter, a gene that makes liver cells glow, to visualise the cells receiving the RNA molecules. This enables us to evaluate the efficacy of our delivery systems and the stability of the mRNA in live animals, reducing the number of animals required to achieve both the efficacy and stability of the RNA. We have also worked closely with a senior biostatistician to develop and implement an experimental design that enables the adjustment of mouse numbers used in a particular group according to the latest information.

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

To minimise the number of animals required for our project, we employ the most up-to-date laboratory methods for processing materials collected from animals used in this license. This allows us to obtain the maximum amount of data from smaller samples and split samples collected from one mouse into downstream applications. By standardising our experimental design and using control animals from one study as controls in others,

we are able to reduce the number of animals required. Pilot studies will be conducted where necessary to inform the design of subsequent studies and potentially reduce the number of animals needed.

In maintaining our genetically engineered mouse strains as homozygous where possible, we reduce the breeding required to generate mice of the required genotype and optimise the efficiency of offspring carrying the appropriate alleles for experimental purposes. As part of standard practice, each experiment will be preceded by writing an experimental study protocol that outlines our objectives and methodology, including statistical endpoints.

These measures ensure we are using the optimal number of animals to achieve the aims of this project. To further reduce the number of mice used, we will implement two strategies. First, we will use the surplus of GEM mice available from the breeding protocol in preference to buying additional wild- type mice. Second, we will reduce the number of breeding steps by administering Nanoformulated CRE mRNA in some situations, omitting the need for crossing mice with strains expressing CRE.

Refinement

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

Which animal models and methods 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 protocols employ only mice as the experimental species. We will use inbred wild-type mice to study the delivery and activity of different nucleic acid molecules, as well as genetically engineered mouse models (GEMMs) that either overexpress a disease-driving gene or have a gene deletion. With these strains, we aim to investigate the delivery of nucleic acids to the depot organs and explore nucleic acid therapy for multiple diseases.

Nucleic acids or peptides will be administered intravenously (IV), intraperitoneally (IP), or subcutaneously (SC). Between administration events and post-administration, mice will be maintained under standard conditions until a specific time, at which point material will be collected and analysed as described in the previous section. These methods are all designed to cause no more than temporary discomfort to the mice.

Our GEMMs model specific diseases that can be treated by peptides, such as diabetes, and allow us to monitor the effect of our mRNA in real time on live animals. This approach reduces the number of animals required for our studies and enables us to assess the activity of our mRNA using methods that limit the suffering of the mice.

To minimise suffering, we adhere to the best practice guidance for the welfare and use of animals in research, currently the NCRI guidelines. Every protocol proposed in this license is the most refined for the purpose and designed to cause the minimum distress and suffering to the animals.

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

Mice are the most relevant species, with the least sentience, that can be used to carry out the proposed research project. The mouse model provides valuable insights into human biology and diseases. The anatomy, structure, and cellular composition of mouse and human organs are similar. For example, basic liver functions, including metabolism, detoxification, bile production, and protein synthesis, are conserved between mice and humans. In addition, many diseases that can benefit from peptide-based therapy, such as diabetes, have mouse models that recapitulate the human disease.

The extensive published knowledge and the array of techniques that enable manipulation of the mouse genome allow access to genetically engineered animals in which to explore the effect of mRNA in specific models.

Therefore, testing the mRNA and delivery systems in this model will be crucial for their ability to be used in disease models and, later on, in the clinic.

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

Our procedures will undergo regular assessments throughout the project to ensure that they are being performed in the most refined manner. Refinements will be made routinely over the course of the project, with the constant advice of veterinary and husbandry staff.

Over the past year, we collaborated with CROs to make several refinements to our procedures. These refinements were intended to ensure that we use the most refined methods for the study and reduce the suffering of the animals. For example, we optimised the maximal dose of some lipid nanoparticles and refined the administration regimen.

In this license, we aim to determine administration regimens that reduce invasiveness while resulting in better drug delivery to depot organs.

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

Work in this licence will be undertaken in accordance with the guidelines published by the NC3Rs and Research Animal Training, which are updated regularly with the best current practice.

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

We will follow updates provided by the National Centre for the 3Rs through receipt of electronic and printed media and by attending the Cancer Institute AWERB committee meetings.

Through this project, we will:

Communicate constantly with veterinary, NACWOs and husbandry staff. Receive updates from the NC3R.

We may attend NC3R and scientific conferences.

These approaches will allow us to be aware of the most recent advances in the field and will enable us to implement these into our research in a timely manner.

13. Assessment of new vaccine delivery platforms in livestock species

Project duration

5 years 0 months

Project purpose

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

Key words

Vaccines, Viral infections, Livestock

Animal types	Life stages
Cattle	juvenile, adult
Sheep	adult, juvenile
Goats	juvenile, adult
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?

We aim to develop new vaccines for viral infections afflicting livestock species including cattle, sheep, goats and pigs.

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

Why is it important to undertake this work?

Vaccines induce protective immunity to infection with viruses. Many viral infections of livestock have a negative impact on the health and wellbeing of animals and can cause

significant financial losses to the farmers raising these animals. Effective vaccines can prevent these effects from disease.

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

Our ultimate goal is to create new, more effective vaccines that will be taken into development and sale to livestock farmers. During the process, we anticiapte new information coming from these studies that will be shared with the research community via peer reviewed publication.

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

This research will result in new vaccines for important viral diseases of livestock. Second, the information generated during these studies will provide new information, approaches, and research tools to other colleauges working on livestock vaccines.

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

Primarily, the results of our work will be described in peer reviewed science publications and at meetings and symposia attended by experts in the field. In addition, we will disseminate the information through the Establishment to our members, mostly livestock farmers. We publish a magazine which shares the outcomes of our research with the public and we routinely describe our work in public forums.

Species and numbers of animals expected to be used

- Cattle: 350
- Sheep: 100
- Goats: 50
- Pigs: 350

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 developing vaccines for food animal species including cattle, sheep, goats and pigs to protect against viral infections that negatively impact on the health and well being of these species. Presently, there are no vaccines for many infections that harm these animals and add to costs for the farmers raising them. Additionally, in cases where vaccines are available but they are inefficient in inducing a sufficiently protective immune response, we are developing new vaccines with better performance.
In general, vaccines will be designed for vaccinating young animals that are no longer protected by maternal immunity to these diseases. We will also test for efficacy of vaccines under development in adult animals to further understand vaccine performance.

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

For the majority of studies, animals will be vaccinated by needle and blood drawn to test ex vivo for immune responses to the vaccine. We also plan to test alternative vaccination systems including intranasal vaccination and using automated injection devices. The latter allows adjustments for delivery in the skin, the underlying dermis or the muscle. These delivery systems have been shown to be particularly effective with some vaccines.

Occasionally we will do live virus challenge to test the efficacy of a vaccine. In these studies, the control, non-vaccinated animals will suffer disease pathology caused by the virus. In addition, if the vaccine fails, those animals will also show disease.

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

The majority of the animal trials will involve testing immunogenicity of vaccines without a challenge with the virus. In these cases, we anticipate the common reactions to vaccination including injection site reaction (soreness or rash). Rarely, animals may show mild symptoms of elevated temperature and depressed appetite or more moderate symptoms of swelling and tenderness at the injection site. These reactions are generally short-lived and have minimal effect on behavior and overall health.

Further, in most of the studies proposed, there is no adjuvant in the vaccine formulation and as such, these reactions are very rare.

When we conduct a live virus challenge trial, the control animals and any animals where the vaccine under study fails, will suffer the symptoms of the infectious disease.

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

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

Most of the animals in these studies are expected to show mild symptoms in reaction to vaccination. This will be approximately 90% of animals enrolled in these studies. Control animals for any of the challenge studies will show moderate symptoms of disease, approximately 10% of all animals used. None of the pathogens we are studying induce severe disease, however, we are sensitive to the possibility that underlying conditions or pre-exposure to other pathogens circulating in these herds could combine with the virus inoculated into the animal for the study and result in unexpected severe disease. If this occurs at all, it will involve less than 1% of all animals used in these studies.

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

- Killed

Replacement

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

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

We are developing vaccines for livestock species. In order to test new vaccine formulations for the immune response to vaccination, we need to inoculate animals and draw blood samples to test whether an appropriate immune response is elicited. As with humans, no alternative to in vivo testing has been developed for analysis of multiple aspects of immune responses to vaccination involving multiple lymphoid organs within the animal.

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

No alternatives exist at this time.

Why were they not suitable?

No alternatives exist at this time.

Reduction

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

How have you estimated the numbers of animals you will use?
The focus of our vaccine development program is infectious diseases of cattle and swine that adversely effect the health and well being of these animals. During the license period, we anticipate testing a least 3 new swine vaccines and 3 new cattle vaccines. Immunogenicity trials will involve small cohorts of animals in pilot studies. Vaccine candidates that show promise in the pilot studies will be tested in larger studies where sample size will be calculated from the pilot study data, yielding statistically significant results. Vaccine candidates that continue to perform well will then be tested in studies where vaccinated animals are challenged with the infectious agent (virus).

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

We test new vaccines in small pilot studies to determine if they are able to induce an immune response. This approach reduces the number of animals enrolled in vaccine trials by eliminating vaccine candidates that do not perform as intended before trials with large numbers of animals are initiated. Only vaccine candidates that perform as designed in the small pilot trials are carried forward.

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

We are developing a number of vaccine candidates where the antigen payload is different and the vaccine delivery system is different. As a result, we plan experiments to use animals for testing one candidate vaccine and then for a second candidate vaccine as the antigens and delivery systems are different, the animals are naïve for the second vaccine

candidate. For example, a cohort of animals vaccinated with our adenovirus vector delivering a bovine respiratory syncytial virus (BRSV) protein will have no immunity to a second test vaccine consisting of a bovine herpesvirus protein delivered by our lentivirus vector. Thus, we can use the same cohort of animals for the different pilot vaccine tests.

Refinement

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

Which animal models and methods 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 most of our animal work, we will vaccinate and give a boost vaccination to the target species and draw blood samples to assess the immune response. In these cases there will be little or no suffering other than the mild discomfort of a needle stick and possible injection site reactions.

In the case of challenge studies with live pathogens, the vaccinated cohorts are anticipated to suffer little or less disease pathology as all vaccines will have been pretested in immunogenicity trials (with no challenge) for induction of effective immune responses. The cohort that receives a placebo (blank) vaccine will show the normal pathology following challenge with the pathogen. These animals will suffer mild to moderate effects, depending on the pathogen being studied.

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

We have established a robust vaccine development program based on testing vaccines in the production animal breeds that farmers use in their operations. Using alternative animals that are different than the production animals on the farm often yields results that are not relevant to the farmed breeds.

Finally, immune responses are challenging to measure under the most optimal circumstances. The time for immune responses to occur and the incubation time of these viral infections is prohibitive for use of long-term anaesthesia.

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

We have generally limited our proposed animal work to vaccination and followed by a boost vaccination and sampling blood to assess the immune response. Thus, we anticipate no suffering other than the mild discomfort of a needle stick, understanding there may be more adverse injection site reactions due to underlying conditions, as noted previously. We will continually seek to further refine our procedures to reduce any welfare costs to the animals.

Animals showing clinical signs associated with a viral challenge will be closely monitored to ensure humane endpoints are adhered to.

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

LASA (Laboratory Animal Science Association) publications/website

LARN (Large Animal Research Network) publications/website

The ARRIVE (Animal Research: Reporting of In vivo Experiments) guidelines 2.0: Updated guidelines for reporting animal research. BMC Vet Res 16, 242 (2020). https://doi.org/10.1186/s12917- 020-02451-y

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

The Establishment is actively involved with the 3Rs program and contribute regularly to information sharing on line and via symposia presentations focusing on the 3Rs. At this time, there are two 3Rs funded projects at The Establishment. Our research group routinely discusses this information, shared within the institute, whenever an animal trial is being designed. Before submitting an animal experiment to the institutional review committee, we seek the input of the attending veterinarian and our animal care staff.

14. Extrinsic regulation of haematopoietic stem and progenitor cells in health, disease and as a therapeutic target

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

Blood stem cells, Leukaemia, Microenvironment, Therapy, Transplantation

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of our Project is to better understand how blood and immune defence cells are produced to improve the outcome of blood stem cell transplantations and the treatment of diseases of the blood 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?

To advance our knowledge on how blood cell production is controlled by its surrounding

environment and to overcome key current limitations in the treatment of cancer, like the resistance of cancer cells to therapies or the occurrence of undesired effects of the treatments, such as a reaction of the cells transplanted to fight cancer against the own body (graft vs host disease, or GvHD).

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

The expected benefits of our programme of work are: a) To broaden our understanding of how blood- forming cells crosstalk to their surrounding tissue in health and disease; b) Dissect the exact role of several cell types regulating blood stem cells; c) Use the new information we acquire to devise new therapies for blood disorders; and d) Explore new or improved therapies for patients who suffer blood disorders or in need of a blood stem cell transplantation.

It is expected that intellectual property emerging from this project will support at least two planned patent applications for a biomarker kit and for therapeutic compounds. These data and intellectual property will support future funding applications and clinical trials to improve the outcome of blood stem cell transplantations and the treatment of blood cancers.

Possible long-term benefits for patients are evidenced by the fact that the blood disorders studied represent a significant clinical need. Acute myeloid leukaemia (AML) is an aggressive blood cancer with poor overall survival (<30% long-term). Curative treatment is arduous for the patient, expensive and requires prolonged hospital stays, representing a significant cost to the NHS and a social burden on the UK. Stem cell transplantation, where stem cells are provided by compatible donors, provides a powerful approach, but is also costly and associated with significant toxicity. Taking these factors into account, AML remains a largely unmet medical need. The myeloproliferative neoplasms (MPNs) are chronic diseases with no cure currently available, except for blood stem cell transplantation, which is only recommended in a minority of patients. MPNs significantly reduce life quality and are costly given their chronic nature and associated complications, such as cardiovascular complications (blood clots or bleeding) or bone marrow scarring impeding normal blood cell production and associated with high risk of developing AML.

Our own work and cumulative evidence indicate that the surrounding tissue (microenvironment) of blood stem cells promotes MPN and AML development, and protects AML cells from current therapies. However, the underlying reasons (which could offer new therapies) are only partially understood.
These diseases are more frequent with advanced age, but whether changes in these tissues during ageing explain their increased incidence in older patients, remains unknown. Our data generated in the previous Project has provided relevant clues. Translation of this research into patients fructified in clinical studies investigating the possible redeployment of available drugs for MPN treatment. The research proposed has the translational goal of increasing the number of patients that could potentially benefit from these therapies. The data generated by our programme of work will contribute to the knowledge related to the blood stem cell environment and potentially offer new therapeutic targets.

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

The short-term benefits will be increased knowledge of 1) the mechanism controlling the division of normal and leukaemic stem cells; 2) how neural-related signals control the

division and activation of blood stem cells and immune cells and 3) why specific abnormal cell-cell interactions occur and why these can lead to blood diseases. The long-term benefits could comprise new methods to prevent severe outcome of blood stem cell transplantation, and/or new therapies (alone or in combination) to improve these outcomes or the treatment of blood cancers.

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

All advancements in our work are immediately circulated within our group on a weekly basis, typically during weekly lab meetings, and on a quarterly basis within the Department. We have several collaborators who are frequently updated with our results, depending on their relevance to their research. Additionally, we present talks and posters in international conferences and publish all our findings in peer-reviewed, open-access journals so that everyone can retrieve this information. Since the peer-review process usually takes some necessary time, for timely report of our findings we will continue to deposit our research timely in public repositories, such as BioRxiv and ResearchSquare. We will disseminate also unsuccessful approaches for the sake of transparency and increased level of information via open access, including platforms such as F1000Research, where we've published before. We collaborate with the pharmaceutical industry to access their expertise, drugs and animal models that can be used to devise therapeutic approaches. Newly devised therapies will continue to be tested by collaborator clinicians through clinical studies.

Species and numbers of animals expected to be used

- Mice: 19,050

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 have been used extensively in blood research because blood production is best established in the mouse. Our group investigates the complex crosstalk between stem cells and their microenvironment, comprised by a multitude of different cell types but also structures (bones, vessels, nerves). It is currently not possible to model this complex microenvironment in the lab.

Our studies will be mostly focused on postnatal stages of blood cell production. Our group is focused in studying blood cancers that occur in adult individuals. Therefore, we will focus our animal studies on adult mice. However, sometimes we need to build the disease model through an early procedure, before the experiment takes place; when that is the case, we will include neonate and juvenile life stages in our studies.

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

We use mice as most of the desired genetic modifications have already been established in genetically-modified mice and our results are immediately comparable with published

literature. When using genetically-modified mice (GAA, Genetically Altered Animals), we will try to use models where we can control the manifestation of disease or harmful observable features (phenotypes) to reduce the time and grade of such phenotypes, which we will closely monitor, score and record in spreadsheets. Most of the mice we use are not expected to present any adverse effects. The vast majority of our procedures will be minimally invasive and are not expected to produce long-lasting harm.

We will mainly use the genetically-modified (GAA) mice bred in house. Occasionally, we will obtain wild type (WT) C57BL/6 or BALB/c mice or GAA mice from external sources. GAA mice comprise disease models for blood cancers, such as acute myeloid leukaemia (AML), myelofibrosis, myeloproliferative neoplasms (MPN), juvenile myelomonocytic leukaemia (JMML), as well as patient-derived xenotransplant (PDX, an animal model of cancer where human blood cells form a patient's tumour or a control donor are implanted into a mouse) and Graft versus Host Disease (GvHD, a systemic disorder that occurs when the transplanted cells attack the recipient's body cells) models. However, in most models the disease induction can be experimentally controlled and surrogate markers (e.g. blood counts) are available in some cases to avoid the development of harmful phenotypes.

In our leukaemia experiments, we try to minimise the time that the animal is sick and during that time, we try to reduce the burden of the disease using therapeutic agents, in line with our research questions.

Mice with a deficient immune system (immunodeficient mice) may be used as recipients for human blood-forming or leukaemia cells. In order to study blood cancers in their full complexity and develop effective therapeutic approaches for tackling them, it is necessary to establish in vivo models. This requires the use of immunodeficient mice, which are capable of sustaining human normal and malignant blood production following transplantation. A minority of the immunodeficient mice (NOD scid gamma, or NSG mice) and derived strains animals in this proposal (up to 25%) will develop an overt cancer. The mice usually develop the disease 2-5 weeks after induction; however, the development of blood cancers cannot be accurately predicted with complete certainty, as it does not always progress in a stepwise fashion. Therefore, after induction, these mice will be closely monitored by regular checks and serial blood counts, which will be recorded in the database and scoresheets. Blood counts allow us to detect and control the development of leukaemia and establish surrogate endpoints before adverse symptoms appear, and therefore we will be able to treat or kill the mouse before overt leukaemia develops.

One of our most frequent procedures is the transplantation of blood-forming stem cells (HSCs), which is the gold standard method to determine their stem cell function. Transplants usually require an irradiation step. Irradiating the animals is the only way to replace the haematopoietic system of a mouse with donor-derived cells. There is no alternative that can generate reproducible results, and efforts have been set to reduce the suffering caused to the animals. By using a split-dose protocol, providing dietary supplements, acidified water and prophylactic antibiotics (after consultation with the named veterinary surgeon), and enrichment to the cages, mouse welfare is significantly improved and re-established after irradiation.

On occasions, we will use treadmill exercise an alternative established and non-invasive model to stimulate the activity of the nervous (avoiding the requirement of drugs). Only moderate exercise will be used, never reaching exhaustion.

In the rare case of invasive procedures with a larger impact, such as terminal bleeds and a subset of surgeries, the animals will be anaesthetised prior to the procedure but will not be recovered after that, and therefore they will not experience any pain.

These studies require the administration of substances (either cell populations in suspension or drugs). When administering a compound, we will use our previous experience and will study the available literature to predict possible side effects and determine the most appropriate dose and administration route. When sufficient information is not available for a specific procedure plan, a pilot study with a reduced number of animals will be designed to assess potential toxicity and efficacy, starting with a low dose through the route with lesser side effects. In pilot studies, or if a substance is being administered to a particular new strain for which side effects may be enhanced, increased health checks will take place for closer follow-up. These measurements will allow to detect an unnecessary harm and to improve endpoints and reduce possible phenotypes.

Rarely, we might need to perform a surgery for an experiment. For example, some experiments require implantation of a subcutaneous minipump or pellet, particularly when a given substance needs to be systemically administered for a long period of time and this approach is considered to be the most appropriate for the animal's welfare. Surgery is also needed to adhere an imaging window to the surface of the mouse skull to be able to image live cells through fluorescent microscopy in the bone marrow. This imaging modality is the only feasible way to study how live HSCs interact with their microenvironment. These experiments are only performed in a highly selected number of strains and mice by highly skilled and experienced researchers, who have optimised the technique to enable a rapid and unremarkable recovery of the animals. These procedures will take place in a room free of pathogens (SPF) in accordance with the Home Office Minimum Standards for Aseptic Surgery and the Laboratory Animal Science Association (LASA) Guiding Principles for Preparing for and Undertaking Aseptic Surgeries, to ensure the least possible distress and lasting harm to the animals.

In a typical experiment, a mouse around 8-week old, commercially purchased or breed in-house, will be added to an experimental group. Any incoming mouse will be allowed to acclimatise to the animal facilities. The mouse will receive a split-dose of irradiation followed by an administration of cells in suspension through a vein. After up to two weeks of recurrent health-checks to prevent the adverse health effects, the mouse will receive drugs (through oral -food pellets- or injection). The mouse will be periodically monitored through small blood samples taken and a variety of blood cell counts will be recorded in the database and scoresheets. These will allow us to detect the early-stages of haematopoietic recovery and/or the initial steps and progression of blood cancer. In blood cancer (leukaemia) models, blood counts will normally reach the necessary threshold 2 to 8 weeks later, when the mouse will be administered the treatment, mainly through the injection of therapy drugs. Upon treatment completion of treatment or disease re-appearance (detectable by the blood counts), the mouse will be killed for tissue analysis.

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

In order to study blood cancers, we need to induce these diseases in mouse models. However, disease manifests by altered blood counts, which normally precede harmful phenotypes and allow to avoid animal suffering. One exception is the mouse model for

GvHD, where transplanted cells react against the recipient body and (like the human disease) affects multiple organs leading to a range of symptoms up to moderate threshold (i.e., diarrhoea, weight loss).

Most of the procedures carried out in this project are not expected to have an impact greater than mild and transient in mice. The main adverse effect expected in our studies is a transient weight loss.
However, the overall impact of this weight loss is minimal due to a close follow-up and provision of dietary supplements, which normally leads to weight recovery after the procedure.

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

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

Based on our experience with similar procedures, the expected severities will be:

Species	Severity	Proportion of animals
Mice	Sub-threshold	20%
Mice	Mild	65%
Mice	Moderate	15%
Mice	Severe	N/A

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

- Killed
- Used in other projects
- Kept alive

Replacement

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

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

Mouse models have been used extensively in blood research and blood cell production is best known in the mouse. Transplantation of blood stem cells is the gold standard method to determine their function, like the capacity of the injected cells to regenerate the blood system of the recipient and give rise to all the different types of blood cells. Moreover, the blood flow, which is key to the study of endothelial cells, as well as the electric pulses originating from the central or peripheral nervous system, are difficult to mimic in the lab. Additionally, our group investigates the complex interactions between stem cells and their home, comprised by a multitude of different cell types but also complex structures (bones,

vessels, nerves). It is currently not possible to model these complex interactions in the lab.

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

We will be implementing non-animal alternatives in this project, as we have already been doing for the past years. We will be using alternative approaches (without involving animal experimentation), when possible. The most frequent and successful alternatives used are optimised culture protocols, such as co-cultures of different cell types. We have improved methods to maintain normal and malignant blood- forming cells cultured together with another type of stem cell found inside our bones (mesenchymal stem cell, which gives rise to bone, fat and cartilage).

In the upcoming studies we aim to further replace animal use through the use of bioengineered models (functionalised scaffolds that can maintain different cell types in culture). We are currently starting a collaboration which will allow us to study some aspects of the bone marrow environment in the lab, using an upgraded version of material and solution that allow us to maintain different types of blood- forming cells in culture, in conditions similar to real life. This sort of Replacement will allow us to reduce the number of animals in some studies.

Why were they not suitable?

Laboratory models cannot replicate the complex signals and interactions that take place in the body; therefore, we cannot limit our research to non-animal alternatives. These alternative approaches are only a suitable option when the research is focused on a particular cell type or interaction. In those cases, we have devised culture conditions that can serve as research models in the lab. However, in most cases the validity of the results would still require confirmation in live animals, for translational purposes.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 based on our previous project and experience, the annual returns of procedures and changes in the mouse lines and the research goals needed.

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

We follow the recommendations under the publication “Guidelines for the design and statistical analysis of experiments using laboratory animals”. We calculate the sample size, taking into account specific response variables, the experimental groups to be compared and the minimum biologically relevant differences to be detected between experimental

groups.

To reduce the number of animals used, we will try to use as many tissues as possible from each animal and store different samples that can be used for diverse assays. Surplus tissues will also be given to collaborators and other researcher scientists (upon request) to reduce the number of mice needed for research. Further optimisation of the use of tissues from the same mice for multiple assays has allowed us to reduce the number of mice originally forecasted in our previous Project.

The number of animals used in the entire programme of work will be minimised by careful planning and scheduling of breeding and experiments and by using the minimum number of animals to answer the question posed. By performing pilot studies and in-depth research on the scientific literature and by developing alternative methods (such as upgraded culture methods that require less cells and animals) we have been able to reduce the number of mice originally forecasted as necessary, compared with our previous Project.

Among all protocols, the ‘Breeding and maintenance of genetically altered animals’ protocol is the one that requires the largest number of animals, although most of these animals have a continued use in the experimental protocols (since the experiments mainly use animals bred in house). For this reason, concerted efforts optimise the breeding efficiency to avoid a surplus of animals. For this purpose, breeding is kept to the minimum for the maintenance of colonies and researchers plan their experiments with sufficient anticipation to increase the number of breeders, if needed. It is normally required to do multiple (2-3) replicates of each experiment for scientific validation. When possible, studies are repeated on the same animals, and the results can be compared with the previous ones, thereby reducing wastage. Some genetically modified mice can be bred in a pure genetic combination background to avoid surplus of animals. However, in most cases mice with combined genetic background will need to be bred, unavoidably resulting in the generation of more animals. When this is the case, animals are offered to other researchers for their possible use.

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

Colony management is performed daily by colony managers and weekly by the principal investigator (Project Licence holder). Mid- and long-term plans of strains are reviewed on a monthly basis. We proactively stop those colonies that are not planned to be used in the near future, and maintain frozen sperm or embryos for strains that are not available commercially or in public collections.

An active communication system within our group and with other researchers, together with frequently updated databases, allow an efficient management, despite the high number of mice and strains required for this Project. We use an online database with all the current animals and the studies assigned to them (or their availability) and an internal chat group for an agile communication regarding mouse colony management. Breeding is carefully planned by researchers well in advance and the future mouse litters have a study already assigned before being born. Surplus animals, mainly due to their unwanted genetic combinations (which are difficult to predict), are allocated for experiments within our group or to other researchers, when possible. Frequently, lab members can make good use of surplus animals for cell obtention (lab studies) or pilot studies, thereby

avoiding the need to obtain additional animals for those purposes. Also, we actively collaborate with other groups to share mouse tissue when possible, to optimise the use of each animal and reduce the overall number of mice used in different projects.

Lastly, we take advantage of pilot studies with a reduced number of mice, which are especially useful when sufficient evidence is not available in the literature, databases, guidelines or from own experience, to assess the procedures and define potential side effects, the smallest drug amount needed to obtain a result, the ideal time points for analysis and the best way to provide the drug.

These pilot studies will ensure the best experimental design, and consequently reduce the overall number of mice used for research.

Refinement

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

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

Using mice as a model system has well-established benefits such as: a short breeding time, large litter numbers, short life span, easy genetic modifications, and easily translation to human therapies.

We use mice as most of the desired genetic modifications have already been established in other labs making ours immediately comparable with the published literature. When using genetically-modified mice, we will try to use models whereby we can control manifestation of disease or harmful phenotypes to reduce the time and grade of such phenotypes, which we will closely monitor, score and record in spreadsheets. Most of the mice we use are not expected to present any adverse effects and the vast majority of our procedures will be minimally invasive and are not expected to produce long- lasting harm.

We will be mainly using the mice we breed, and occasionally we will purchase wild-type mice from commercial sources. Genetically modified mice comprise disease models for blood cancers and GvHD. However, in most models the disease start can be controlled and the blood samples taken frequently tell us when the disease starts so that we can reduce animal suffering by treating or killing the mice at that time.

In our blood cancer animal experiments, we try to minimise the time that the animal is sick and during that time, we try to reduce the burden of the disease using therapies, in line with our research questions. Pain management will include the use of medicated palatable substances for voluntary treatment such as flavoured jelly, paste or milkshake liquid.

Mice with a genetically-modified immune system may be used as recipients for human cells. In order to study blood cancers in their full complexity and develop effective

therapies, it is necessary to establish humanised animal models. This requires the use of mice with a defective immune system, which are capable of sustaining human blood cell production after human blood stem cell transplantation.

One of our most frequent procedures is the transplantation of blood stem cells, the gold standard method to investigate these cells. Transplants usually require an irradiation step. Irradiating the animals is the only way to replace the blood system of a mouse with donor-derived cells in an efficient, reproducible manner that can be compared with previous experiments and the large scientific literature. There is no alternative that can generate reproducible results, and efforts have been set to reduce to the minimum any suffering that may cause to the animals. By using a split-dose protocol, providing dietary supplements and enrichment to the cages, mouse welfare is significantly improved and re-established after irradiation.

On occasions, we will use treadmill exercise an alternative established and non-invasive model to stimulate the activity of the nervous system (avoiding the requirement of drugs).

In the rare case of invasive procedures with a larger impact, such as terminal bleeds and a subset of surgeries, the animals are anaesthetised prior to the procedure but not recovered after that, so that they will not experience any pain.

Also, all our studies imply the administration of substances (either cell populations in suspension or drugs). When administering a drug, we rely on our previous experience and the literature to know its expected undesired effects and determine the most appropriate amount and way to provide it. When sufficient information is not available for a specific study plan, a pilot study with a reduced number of animals will be designed to assess for potential toxicity and efficacy, starting with a low amount through the route with lesser undesired effects. In pilot studies, or if a drug is being administered to a particular mouse strain for which side effects may be enhanced, increased health checks will take place for closer follow-up. These measurements will allow to detect an unnecessary harm and to improve endpoints and reduce possible suffering.

Rarely, we might need to perform a surgery for an experiment. This might be to either install a minipump or pellet under the skin, if a substance needs to be provided for a longer period of time and this approach is considered to be the most appropriate for the animal’s welfare. Surgery is also needed to implant an imaging window in the mouse skull to visualise blood stem cells in the animal, the only feasible way to study the interaction of blood stem cells with their home in real time. These experiments are only performed in a highly selected number of mice by highly skilled and experienced researchers who have perfected the technique for the animals to ensure a rapid and unremarkable recovery. Also, all our procedures are done in the cleanest possible conditions and in accordance with the Home Office Minimum Standards for Aseptic Surgery and the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgeries, to ensure we cause the least distress and lasting harm to these animals.

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

Mouse models have been used extensively in blood research because blood production is best understood in the mouse. Given the time needed for blood regeneration after transplantation or for blood cancer development, it is not possible to use immature life stages or terminally anaesthetise a mouse for the duration of the study.

Additionally, our group investigates the complex interactions between mammalian blood stem cells and their home, comprised by a multitude of different cell types but also structures (bones, vessels, nerves). It is currently not possible to model these complex interactions in less sentient (and less complex) species.

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

Experience has allowed us to refine our animal models and methods. Our current protocols are optimised based on our results and the feedback from the animal facility senior technicians, which allows us to reduce the impact on animal welfare while obtaining the necessary data from each mouse. Mice are housed with cage mates, in an environment with added enrichments such as bedding materials, tubes or playing items, to reduce their stress and ensure their socialisation and entertainment.

Furthermore, all mice undergo regular health checks adequate for each protocol and step. Early detection of any health concern allows us to make appropriate plans for those animals to prevent a worsening that could compromise their welfare, as well as to increase their monitoring. Health concerns are registered in an online database, which allows us to detect any possible unexpected side effect (or a major frequency or intensity for a specific mouse strain) and adapt the other studies accordingly to minimise the harms for the animals.

Mice with a genetically-modified immune system may be used as recipients for human cells. In order to study blood cancers in their full complexity and develop effective therapies, it is necessary to establish humanised animal models. This requires the use of mice with a defective immune system, which are capable of sustaining human blood cell production after human blood stem cell transplantation.

To generate transgenic mice, methods where any symptoms can be experimentally controlled, will be used when possible. The mice will be allowed to acclimatise to the animal facilities and should not display any symptom until we trigger the disease initiation. Most of the strains we use are not expected to present any adverse effects and the vast majority of our procedures performed are going to be minimally invasive and are not expected to produce long-lasting harm. When unexpected symptoms arise (such as some deformities of the skeleton), the animals will be monitored and scored using sheets.

We find that strains that have been crossed with Nestin-gfp mice have an increased incidence (<5%) of developing skeletal deformity. The skeletal deformity appears as a “hunch” in the spine, sometimes accompanied by a waddled walk, most noticeable in the hind limbs. Most often, skeletal deformity is observed in older animals and more rarely in young animals. We are trying to breed this phenotype out of our colony by periodically refreshing breeders with wild-type mice. The animals with skeletal deformity will not be used as breeders but, if the deformity is mild, they might be used for downstream experimental protocols, to reduce the number of mice generated. Animals developing skeletal deformity will be recorded and monitored. We will health check the animals on a weekly basis and record their follow up in an observation sheet, to see if their overall health is affected (weight loss, impaired gait or reduced mobility). If the animal starts to lose weight or worsens the health checks will become more frequent, up to daily ones if needed. Mice will be killed by a schedule 1 method if they lose 10% of peak body weight.

In our experiments with animal models of blood cancer, we try to minimise the time that the animal is sick and during that time, we try to reduce the burden of the disease using therapies, in line with our research questions.

In the case of irradiation, we try to alleviate collateral damage and prolonged suffering by splitting the irradiation dose and to reduce the risk of infections and improve recovery rates by providing acidified water and prophylactic antibiotics (as advised by the Named Veterinary Surgeon, or NVS). Depending on the compound, pellets might be used for chronic studies as an alternative to drinking water, which can negatively affect the animal's weight. In those cases, the animal's weight will be carefully and frequently monitored and recorded.

On occasions, we will use treadmill exercise as an alternative established and non-invasive model to stimulate the activity of the sympathetic nervous (avoiding the requirement of drugs). Only moderate exercise will be applied, never reaching exhaustion.

To reduce procedural harm, we will ensure that researchers will receive extensive training and only perform procedures when they have achieved competency, which will be carefully monitored and logged in databases. When appropriate, researchers in training will practise on dead animals and inanimate objects before handling live animals after having watched videos demonstrating the technique and experienced personnel. All researchers will be supervised until they are assessed as competent.

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

We will follow the PREPARE guidelines for planning animal experiments and improving reporting. These complement the ARRIVE guidelines containing the best practice and important information to include in publications describing animal research.We will adhere to the Guidelines for the Welfare and Use of Animals in Cancer Research as set out by Workman et al. 2010 and implement the principles in the NC3Rs guidelines (including justification of species, details of power calculations and plans to minimise experimental bias). We will also follow the UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia, including leukamia.

We will consider guidance from the Laboratory Animal Science Association, (LASA) https://www.lasa.co.uk/current_publications/ and will follow the Animal testing and research: guidance for the regulated community published by the Home Office on 26th March on 2013 and updated on 20th Oct 2022.

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

Both Project and Personal License holders will keep up to date about advances in the 3Rs by frequently consulting broadly accessible resources such as the Home Office guidance (https://www.gov.uk/guidance/research-and-testing-using-animals), the Laboratory Animals Science Association website (https://www.lasa.co.uk), NC3R's (http://www.nc3rs.org.uk), RSPCA and UAR (http://www.understandinganimalresearch.org.uk/). Locally, we have access to updated information on Policies, AWERB, 3Rs Search Tool and training.

We organise weekly lab meetings and journal clubs to ensure that we keep up to date within relevant experimental animal work in our research field. Not just by following the latest publications, but also by taking part in reviewing articles and joining international and national conferences, we can keep track of the newest approaches other groups might be doing regarding animal research and ensure our methods stay updated within the new refinements implemented by others.

I will continue to participate in 3Rs workshops intended to guide other researchers on the use of substances that we frequently administer, and the optimisation of these protocols to minimise animal suffering.

15. Gut hormone modulation of metabolism

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Enteroendocrine hormones, Gut-brain-pancreas axis, Diabetes, Obesity

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to characterize how gut hormone secretion is controlled and how gut hormones modulate metabolism and body weight.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 Diabetes are major diseases putting considerable strain on health services worldwide. Patients suffering from obesity, definable as excess storage of fat, and/or diabetes, definable as failure to control blood glucose adequately, often develop complications affecting their hearts, blood vessels, liver and kidneys, leading to organ failures, amputations and early death. Modulating eating behaviour, energy expenditure and blood glucose handling are obvious therapeutic interventions for obesity and diabetes and there is good evidence that gut hormones modulate nutrient handling and eating behaviour. An example is glucagon-like peptide-1 (GLP-1), which is secreted from special cells in the intestine during a meal. Analogs of GLP-1 are relatively frequently in the News,

as they have been approved for the treatment of obesity by the NHS in March 2023, adding to their well established role in the treatment of diabetes. We are investigating how GLP-1 secretion is initiated during a meal and which cells this hormone targets to reduce appetite. There are about twenty different gut hormones, many of which also affect eating behaviour. New drugs are under development which combine the action of GLP-1 with some of these other hormones, promoting even better weight loss in pilot studies. Our research aims to understand what mechanisms underlie these better outcomes and if and how we might be able recruit the bodies own spare capacity of hormones to treat obesity and diabetes.

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

We expect to gain new insights on how gut hormone secretion is controlled and how in turn these hormones modulate metabolism and eating behaviour. We expect that we will continue to publish our findings in open access scientific journals and at scientific conferences. We also have established collaborations with the pharmaceutical industry, which is actively developing gut hormone based therapies for the treatment of diabetes and obesity. Any new mouse models developed during this project will be shared with the scientific community, as will be data arising from the project, by uploading the data to appropriate data depositaries.

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

The scientific community and pharmaceutical industry is actively developing gut hormone based therapies for obesity and diabetes. Our findings should guide these attempts and clarify the mechanisms of action these new therapies employ. This might open new avenues of development or highlight potential risks, such as unexpected and unwanted side effects. On the longer timescale we hope that new and effective treatments will be available for patients - glucagon-like peptide-1 (GLP-1) based therapies are already licensed and newer therapies combining GLP-1 action with that of other gut hormones have shown promising improved therapeutic outcomes in pre-clinical and pilot studies. This project will explore how these better outcomes come about and hopefully identifies further improved combinations. Our group is aware of at least one company developing agents that target GLP-1 secreting cells in the intestine - our research should help to inform and improve these attempts.

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

We will continue to publish in open access peer reviewed scientific journals, including, if appropriate, open access publishing platforms which do not employ editor based publication decisions, but allow ongoing peer review of deposited data (such as F1000Research), and participate in scientific meetings. We will continue to collaborate widely, sharing mouse models and ideas internationally, as evidenced by our collaborative publication record. The group is involved in outreach programs to the wider public, such as school visits - although only loosely connected to the specific questions asked in this project, this raises a wider awareness of how the gut affects body weight and health; we hope this to have further impact by promoting healthier eating choices.

Species and numbers of animals expected to be used

- Mice: 18050

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 as a mammalian species are close in their regulation of metabolism to humans. The suitability of mice to explore the role and ability of gut hormones to modulate obesity and glucose handling is exemplified by the successful development and subsequent translation of GLP-1 based therapies. We will predominantly use adult mice (>6-8 weeks old) for our experiments. Given that one focus of our research is on the regulation of feeding behaviour by the central nervous system this is the stage at which the underlying neuronal circuitry is established and cross talk with gut hormones (either arising from the gut or being released locally within the brain) can be studied. In addition, mice genetics are very advanced and it is possible to perform bespoke and restrictive manipulations of rare cell types, like the cells releasing gut hormones, which are found scattered in the gut lining and only constitute about 1% of the gut lining, or their neuronal targets.

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

To be able to identify and/or manipulate the rare cell types underlying the gut brain axis, we need to produce transgenic animals that tag the cells of interest, gut hormone producing cells in the gut and neurons in the central nervous system, either expressing the same hormones as neuromodulator or the receptors for these hormones. Many animals will be bred for this purpose and not undergo any further regulated procedures - some will be used as tissue donors for experiments performed on tissues harvested after they have been killed, as for example for the preparation of brain slices, in which we will monitor neuronal activity through electrical recordings or live cell imaging.

Animals used in regulated procedures might need additional modifications before they are assessed. This might involve feeding of altered diets to make them fat or lean and application of drugs, which induce or silence a genetic alteration or are considered to target the cells along the gut brain axis; these will typically be given through the drinking water, food or injected, typically directly into the blood stream through a vein, or into the body cavity or under the skin. Some animals will also be surgically modified. This is needed for example to manipulate specific areas in the brain, either through targeted injection of viruses into this area, thereby avoiding the delivery of a specific genetic alteration to other areas in and outside the central nervous system or through similarly targeted delivery of cell manipulating agents. Brain surgery is performed under general aneasthesia on specialist frames aiding the correct positioning of the agents and takes typically less than 30 minutes, with mice usually recovering to normal behaviour within 2 hours. Cell manipulating agents include for example gut hormones and viruses but also light in mice which express light sensitive ion channels to modulate neural activity or light sensitive sensors that allow us to monitor intracellular components such as calcium, as good measure for neural activity. In cases where a administration of agents (for example light) will have to be repeated frequently, a permanent access to the brain is implanted and mice will be single housed after surgery to prevent them from damaging each others access. Most mice will only undergo a single brain surgery, but some viruses used to

identify the neural circuits a specific neuron sits in require two surgeries typically with a 3 week interval between surgeries. A minority of mice will undergo surgery aimed to interfere with known neuronal pathways connecting the intestine with the central nervous system performed under general anaethesia and typically lasting 10-20 minutes; most of these will not also undergo surgery to the central nervous system, but a few will, which then would typically be performed at the same time. Some mice will be surgically altered to enable direct infusion of nutrients and other enteroendocrine cell modifying agents directly into the intestinal lumen, bypassing the mouth and/or the stomach. These surgeries will be performed under general anaesthesia with adequate pain relieve and animals will be closely monitored for the rest of their lives with frequent flushing of the artificial access tubes to prevent complications due to food entering from the intestine getting stuck and blocking the tubes. We expect animals to recover from this surgery within a few hours and behave normally - a minority of animals might undergo this surgery in conjunction with brain surgery in which case we will aim to combine both surgeries.

Once prepared, animals will be tested for metabolic and behavioural parameters. In the most simple experiments we will measure their food intake and body weight whilst being fed special diet, for example containing a high fat content, which mice usually prefer to their standard diet. For this animals might be fasted for a few hours before the food intake quantification and single housed. Animals might undergo metabolic fitness assessment - for example their glucose tolerance will be assessed either by administering a glucose bolus intraperitoneally or given through a tube directly into their stomach at a defined time. Typically such tests will be performed whilst agents that modify gut hormone release or act on gut hormone receptors are also administered, typically directly into the blood stream or into the main body cavity. Occasionally longer term administration will be achieved by placing slow release devices under the skin under anaesthesia before the metabolic assessment. Metabolic fitness test like these will typically involve taking small amount of blood from superficial veins to be able to monitor blood glucose and/or hormone levels.

Some animals will be placed into specialist equipment to measure food intake and energy expenditure in which they are typically single housed for 3 days, including 1 day adaptation to the new environment. These "calorimetric chambers" resemble the home cage environment the mice normally life in and mice appear to be happy in these for days without obvious signs of stress. Sometimes we will assess the body composition of animals for which they will be placed in specially designed tubes made of material identical to that found in the home cage to enable them to remain still and secure and quickly have a scan without the need for sedating drugs. Animals will also be trained to perform task in "operant chambers" - these are modified cages in which mice can work for treats (for example poking a computer touch screen), which will be provided in small quantities through special dispensers, whilst the mouse is monitored through video cameras. Training for these typically will take several daily sessions over a couple of weeks and typically individual training sessions will not last more than 2-4 hours; however, on occasions animals will be kept in these operant chambers for longer, in which case we will provide extra housing and enrichment material to simulate a home cage environment as much as possible and food and water provision, whilst intake is monitored, will not be limited to task performance. Some mice (typically whilst in these operant chambers) will be linked to light guides enabling light driven activation or inhibition of specific neurons in the central nervous system or neuronal activity monitoring whilst performing feeding tasks. Other mice, which express designer receptors exclusively activated by designer drugs (DREADDs), will typically be injected with vehicle or designer drug on different experimental days (cross-over design) whilst performing these tasks. Other test mice will

undergo involve conditioned taste aversion or preference in which mice are given the choice between different flavoured liquids or meals which are paired with gut hormone releasing or gut hormone receptor modifying drug administration or control agents known to induce aversion or preference.

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

Most animals will experience no more discomfort than that experienced by any rodent bred in captivity and residing in a modern animal facility.

Some animals will experience transient (seconds) discomfort when given injections or when having blood samples taken. The injections will often be of naturally occurring hormones, or compounds closely related to them. On occasion, animals may be given compounds that are recognised to produce circulating levels of hormones that are seen in acute illness. These may reduce the animals' drive to seek out and eat food in the hours after they have been given.

When given a different diet or treatment, some animals will gain or lose weight. This will be within closely monitored parameters that take into account other important aspects of their appearance and behaviour. This weight change will typically occur slowly over weeks.

A minority of animal will also undergo surgery that will require a general anaesthetic. Inevitably, as with any operation, animals will have some discomfort in the immediate hours after the operation at the site of the incision. However, this will be minimised by administration of painkillers under instruction from a veterinarian. The general anaesthetic needed for this surgery may also make the animals less active and less hungry in the first day after the operation but we expect them to recover their appetite and vitality within 48 hours. Animals undergoing brain surgery that leaves access tubes for subsequent substance administration of light monitoring will need to be single housed to prevent them from dislodging each others access tubes - they will be closely monitored and in our experience they show normal behaviour without obvious signs of distress.

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

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

Sub-threshold - 40%
Mild- 30%
Moderate- 30 %

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 are researching the gut-brain axis. We are particularly interested in hormones released by the intestine after a meal which not only affect how the gut handles digestion of food but also affects future food intake by modulating hunger and fullness perception. There are already new therapies for diabetes and obesity based on the actions of one of the gut hormone glucagon-like peptide-1 (GLP-1), with other experimental medicines currently being developed that combine GLP-1-action with other gut hormone activities; these show promising better weight loss in experimental animals and human volunteers. To develop these further we will need to understand better i) how release of the different hormones is regulated in the intestine and the central nervous system, where some of the hormones are also made and ii) where and how they act to change feeding behaviour and/or energy expenditure, neither of which can be assessed in vitro.

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

To understand how hormone release is regulated in the gut we have now mostly moved to intestinal organoids - these are cell cultures of just the intestinal epithelium that can in principal be kept indefinitely and appear to give rise to all gut hormone secreting cells. We have established mouse and human derived intestinal organoids is which we have genetically labelled different intestinal hormone secreting cells with fluorescent proteins. These allow identification, characterisation and manipulation of stimulus-secretion coupling pathways within the hormone secreting cells themselves and also to investigate cross-talk between different cells in the gut epithelium.

However, they lack the other tissues interacting with the epithelium, such as the enteric nervous system and currently there are no in vitro models that recapitulates these complex interactions. We also for example find that glucose-triggered GLP-1 secretion is totally dependent on a particular transporter in intestinal organoids preparations, but that mice lacking this transporter nevertheless elevate GLP-1 levels in their blood in response to glucose in the diet, a mechanism that might be exploitable to stimulate GLP-1 secretion therapeutically, but can currently only be explored in live animals.

To understand how gut hormones affect feeding behaviour we need to measure behavioural outcomes, which is not possible with isolated cells in a dish. We are using acute brain slices made from mice killed for this purpose to monitor which neuronal circuits are modulated by gut hormones, however, to link these to behavioural outcomes we also need to perform experiments in freely behaving animals.

Both, acute brain slices and cultures of differentiated neurons can also be used to characterize what a gut hormone might affect within a single neuron. To some extent the connectivity of neurons underlying behavioural pattern generation can also be investigated in brain slices, but many longer distance connection are lost, whereas connections in primary neuron cultures do not adequately replicate connectivity found in the brain.

Why were they not suitable?

See above - intestinal organoids are very powerful and we use these, but outcomes need

to be further verified in animal experiments and more complex mechanisms like the unexpected GLP-1 response to dietary glucose when glucose uptake in the intestine is inhibited, are not well modeled.

Neuronal cultures can be used to identify molecular signals within a neuron, but lack the connectivity needed to produce specific behavioural outcomes. Connectivity and cross-talk between neurons can to some extent be explored in brain slices isolated from animals, but behaviour has to be explored in freely behaving 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 are interested in physiological outcomes, so are interested in robust effect sizes. In most cases we are able to observe effects in group sizes of 8 and whenever possible we use a cross-over design in which each mouse is its own control.

Often we need to bring together different genetic alterations (usually at least 2). Each animal has two copies of each gene and for some experiments both copies need to be altered. To bring these together with genetic alterations in a different locus is often challenging and produces many animals which only partly have the desired genetic change - such animals are killed early in life, when they cannot be used as controls for experiments or for future breeding, but contribute to the number given above.

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

I consulted the NC3Rs experimental design assistant and we discuss all animals experiment within our group. Our field is rapidly evolving and experimental designs are refined through discussions with both our collaborators and competitors directly or through the literature. For example until fairly recently glucose tolerance was frequently assessed after prolonged fasting of mice, but a newer consensus takes into account that mice can go into torpor after prolonged fasting. Torpor is a state of usually reduced body temperature and metabolic rate, enabling mice to survive times of food scarcity or famine. As this is not likely a good model for the physiological states we want to treat in obesity and diabetes, most groups are now fasting the mice for no longer than 16 hours and ideally only for 4-6 hours - whilst this has no direct impact on the group size used in a particular experiment, the better data quality achieved indirectly reduces 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?

Our research is shaped by guidelines for animal research, such as PREPARE and

ARRIVE.

Whenever possible we share tissue within our group and with other groups in our institution. However, many of our animal models bring together several genetic alterations enabling specific manipulation or observation of a specific subset of cells within the intestine or the brain and therefore do not qualify as adequate wild-type controls.

We are using the Cre-lox system; Cre is a recombinase that changes the expression of "f-lox-ed" genes when expressed; by putting the expression of Cre under the control of specific promoters we can therefore modulate "floxed" genes just in cells that would normally express from this promoter, for example a promoter that drives the expression of a gut hormone. By sharing floxed reporter genes between different Cre-lines we are able to keep breeding pairs of these different Cre-lines low whilst still minimizing the risk of genetic drift due to excessive inbreeding. Cre-negative offspring can be used to cross-breed or as controls for Cre-positive animals.

Refinement

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

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

We use genetically altered animals in which rare cells either in the gut lining or in the brain are specifically labeled and manipulatable. These genetic alterations have no impact on the animals wellbeing.

Some animals are surgically modified to allow live monitoring and/or manipulation of these cells during behaviour. We will continue to work with our colleagues and collaborators to improve these so that they inflict as little harm to the animals as possible. All animals will receive pain relieve medication after surgery. Surgery will be performed aseptic and under general anaesthesia, if this is beneficial for the procedure (minor surgery, like the placement of a slow release pharmaceutical agent, will be performed with local anaesthesia).

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

To understand the gut brain axis and develop new modifying drugs useful to treat diabetes and obesity in humans we need to work in a species that has a similar physiology to humans. Mice have a proven track record, with research on the GLP-1/GLP1R axis having translated well into treatments now widely used in type2 diabetes and obesity therapy.

As we are interested in behavioural outcomes we cannot use terminally anaesthetised mice, as these are not showing any feeding behaviour driven by nutrient availability or by pleasurable experiences in response to eating, which are strongly influenced by the gut

brain axis we wish to understand better, so that on the long run we will be able to exploit this therapeutically.

We need to work in mature mice, as only in adult mice the underlying neuronal circuits and feedback mechanisms are fully functional.

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

All our animals receive close postoperative monitoring and analgesia as required and are checked frequently (initially hourly and later daily after surgery). For example, post central nervous system surgery postoperative analgesia is provided as standard. For other procedures with potential to induce pain (for example stimulation of serotonin producing EC-cells, which have been implicated in gastrointestinal nociception) we will use pain scoring schemes like for example the McGill Grimace scheme and postural/behavioural clues and provide analgesia as required. We will also use scoring sheets rating animals body condition (for example BCS1-5 scores defined in the application) rather than relying solely on body weight changes, as substantial loss of body weight is a desired outcome in diet induced obese mice. These will also be used for example when new diets, potentially resulting in neophobia (the fear of all new things often seen in mice refusing to eat unfamiliar diets), are introduced. When animals are exposed to new diets or environments they receive training sessions.
New diets are usually introduced as an add on before a total switch, which we find reduces neophobia and increases the acceptance of diet switches.

Behavioural arenas ("operant chambers") are introduced in short training sessions, with the animals returning to group housing when this is possible or at least to their home environment before data is collected in subsequent experimental sessions once the animals are familiar with the setting. Whilst the behavioural arenas have a flooring that allows excrements and wasted food to fall through and out of reach, which is necessary to adequately monitor feeding behaviour and motivation, the floors are not wired grids, but fully weight bearing (small holes punched into a flat surface). When animals are housed for more than 4 hours in these arenas we will provide housing with nesting material to which the mice can retreat, and other environment enrichment components provided as standard in our home cages (e.g. wooden gnawing sticks), attempting to replicate a home cage environment as much as possible. To minimize disturbances during the experiment individual behavioural cages are in noise cancelling cabinets and mice are monitored through video cameras.

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

We are part of a very active scientific community that tries to evolve and refine methods constantly. New methods and method modifications are shared through peer-reviewed publications and at meetings, either during collaborations or at conferences. We expect that methods will be refined further during the duration of this project license. We will also aim to implement guidance provided by the Laboratory Animal Science Association, (LASA; https://www.lasa.co.uk/current_publications/) and other sources of 3Rs advice as they arise during this project.

How will you stay informed about advances in the 3Rs, and implement these

advances effectively, during the project?

Our institution runs a 3R enquiry email group, which informs about updates and developments and has in recent years implemented an animal and method sharing platform. In addition group members are encouraged to consult open resources, such as https://nc3rs.org.uk/3rs-resources or https://norecopa.no/databases-guidelines.

16. Humoral and cellular immune mechanisms of peripheral neuropathies and neuropathic pain

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

Antibody-mediated pain conditions, Immune-mediated neuropathies, Nerve injury, Neuropathic pain, Sensory 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 aim of this project is to investigate the role of antibodies and immune cells in the development and resolution of pain in peripheral nerve disorders. We will do this by modelling traumatic nerve injury and immune-mediated peripheral neuropathies.

Traumatic nerve injury models are designed to have relevance to lumbar radiculopathy (i.e. ‘sciatica’), nerve compression, surgery-induced nerve damage and other accidental causes of painful nerve trauma. Immune-mediated peripheral neuropathy models are designed to have relevance to inflammatory neuropathies, neuropathies associated with immunotherapy, autoantibodies targeting the nervous system, as well as diseases with putative autoantibodies such as small fibre neuropathy and fibromyalgia.

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

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

Why is it important to undertake this work?

Chronic pain caused by damage or disease of the nervous system (known as neuropathic pain) will affect as many as 10% of adults in their lifetime. Although strong pain killers are helpful in the short term, their efficacy lessens over time and their use comes with a risk of serious side effects such as a dependency on opioid drugs.

We know that the immune system plays an important role in responding to nerve damage. This gives us an opportunity to intervene immunologically in the failure of some people to recover from nerve injury and the ensuing neuropathic pain.

However, the immune system is complex, and in some cases may even contribute to diseases of the peripheral nerve. It is therefore important for us to understand how each of the different parts of the peripheral immune and nervous systems fit together as a whole.

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

The main output of this work will be publications focused on determining the mechanisms underlying pain in clinically-relevant models of nerve injury and peripheral neuropathies involving the immune system. As we develop new treatments for other diseases, such as cancer, patients can be left with chronic debilitating pain. Therefore, this work will also help to ameliorate treatment side effects that impact on peoples' lived experience post-cancer.

The specific outputs are expected as follows:

Profiling the immune response to painful and painless nerve injuries.

Identification of immune factors related to the development and resolution of pain after traumatic nerve injury.

Understanding the mechanisms of pain in peripheral nerve conditions caused by the immune system.

Validation of immune targets documented in clinical patients and human biobank samples in order to develop therapeutic agents for pain.

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

Publications are the core focus of any academic work and should be expected throughout the project.

In the short term, this work will strengthen the peripheral neuro-immune research field and will build the case for further research into novel therapeutic targets.

In the medium term the findings from this project may guide alternative treatment options in the pain clinic using existing approved immune interventions.

In the long-term, the goal of this project is novel medicinal development, which may not be realised until beyond the lifetime of the initial project licence.

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

Research outputs will be shared at scientific conferences and published in open access journals in accordance with funder policy. Licensees will be encouraged to publish negative results in suitable journals so that animal work is not needlessly repeated by others.

For the duration of this licence, the use of tissues obtained from experimental animals will be maximised by banking in cryostorage and used repeatedly where possible, in addition to sharing with colleagues and collaborators where appropriate.

This project is designed with collaboration in mind, locally within the institute, as well as across the UK and internationally. The project aims to share findings beyond the immediate scientific field of peripheral neuropathy, as well as across the neuroscience and immunology fields.

Species and numbers of animals expected to be used

- Mice: 14,000

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.

Rodents are the most commonly used animals for the study of neural injury and persistent pain because of the vast knowledge of the rodent peripheral nervous system. Sensory and motor neurons in the rodent have comparable features to those seen in primates, including humans, and show a similar response to nerve injury.

This project requires the use of mice specifically for three reasons:

The immune system of adult mice contains the vast majority of immune cell populations and factors that are found in humans.

A large library of genetically altered mice exists for investigating the immune mechanisms of neuropathic pain.
Mice are sufficiently sentient to perceive both evoked and spontaneous pain that can be assessed with behavioural tests, the treatment of which is the ultimate goal of this project.

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

Typically, animals will experience mild, transient pain and no lasting harm from administration of substances by injection using subcutaneous, intraperitoneal, or intravenous routes, followed by various behaviour tests where they will be exposed to

environmental cues to assess the level of pain or discomfort (Protocol **4**). Where administration is required for prolonged periods, animals may be surgically implanted with slow-release devices such as a mini-pump.

Mice may undergo an injury to a peripheral nerve by either the nerve being cut or crushed whilst under general anaesthesia (Protocol **3**). These animals will experience some discomfort after surgery and some mild to moderate pain which will be treated with analgesics at the time of surgery. Once recovered from surgery the mice will start to undergo a series of brief exposures to stimuli such as touch, heat and cold to assess the affected limb.

One or more blood samples may be collected before or after nerve injury surgery or substance administration. Typically animals will experience mild and transient discomfort from blood sampling. In a subset of experiments, mice will undergo whole body imaging to trace immune factors over time.
Nerve function data may be obtained under terminal anaesthesia at the end of experiments.

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

Some treatments, such as those that modify the immune response of the mice, may induce a short period of inactivity, reduced appetite and modest weight loss, similar to that seen with patients undergoing immunotherapy. This effect is expected to last a few days and no more than one week. During treatment with substances mice will be closely monitored and provided with additional home cage and nutritional support by placing palatable food/gel within easy reach and providing nestlets instead of sizzle nest, which support temperature regulation for animals showing temporarily less activity.

The nerve injury procedure will inevitably result in some level of pain and discomfort, including that from the surgery itself. Similar to patients in hospital, mice will undergo surgery under general anaesthesia. Analgesia will be given before and after surgery to reduce the pain in the immediate period of recovery following surgery. Mice will recover from surgery in a warm cage in isolation before being returned to home cage after recovery (30-60 min).

Nerve injury will only be carried out on a single limb, meaning the mouse will have the ability to avoid putting pressure on the affected limb. Some weakness will likely occur but should not prevent the animal moving around the home cage, feeding, grooming and interacting with cage mates. Full motor coordination will return within 2-3 weeks. In some types of injury (e.g. partial crush) the pain-like behaviour is expected to last more than two months but should not develop into more severe impairment of limb function and animals can be housed for the duration in their home cage with normal animal husbandry. For mice with painful nerve injury (approximately 50% of animals undergoing surgery), behaviour testing is likely to result in brief but escapable, mild pain sensation, with fluctuating mild ongoing pain.

For some experiments, mice with genetic alterations will be used to understand the interactions between the immune cells and sensory neurons. These genetic alterations may make the mice more or less susceptible to pain, or to the effects of immune modulators. In such cases, immune or neuroactive substances will be tested in smaller

doses in pilot studies to ensure animals do not experience unexpected pain, suffering, distress or lasting harm.

At the end of the experiment mice will be humanely killed.

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

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

25% Subthreshold
25% Mild
50% 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?

Adult mice have a fully developed somatosensory system with a diversity of neuron types and the vast majority of immune cell populations and immune factors that are found in humans. This will allow the study of the mechanisms of neuropathic pain development and resolution after nerve injury with the closest parallels with the human condition that allows for practical and ethical intervention.

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

In keeping with the principals of the 3Rs, experiments into the mechanisms underlying neuro-immune interactions are replaced where possible by in vitro modelling systems, significantly reducing the numbers of animals used.
For example, our laboratory is pioneering the use of co-cultures of sensory neurons and immune cells from mice, as well as humans using stem cell technology, to study how antibodies and immune cells physically interact with nerve cells in health and disease.

The use of human induced pluripotent stem cells (iPSC) in particular to derive sensory neurons for molecular and functional assays serves to further replace the use of animals and strengthen the translation of findings to humans.

Why were they not suitable?

Cell culture can tell us about the molecular interplay between the immune and sensory nervous systems, and even the effect on nerve cell activity. However, these cell cultures are designed to ask complex questions in a simple system of direct cell-to-cell interactions.

On the other hand, understanding the effect of the immune system on nerve structure and health, and ultimately pain perception, requires the complexity of the intact organism and all the potential indirect interactions between immune and nervous system function.

The complexity of neuro-immune interactions in the response to nerve injury, the changes in the nervous system leading to neuropathic pain, and the effect of immune manipulation, can therefore only be observed in the whole animal.

Nevertheless, our in vitro models provide a powerful method to further probe the immune mechanisms revealed in our focal nerve injury and immune-mediated neuropathy models in mice. They also offer translatability between mouse and human culture systems, paving the way for biomarker investigations in patient tissue samples. In vitro modelling will also be used to select the most effective immune reagents prior to testing in our animal models, reducing the need for multiple cohorts of mice in therapeutic proof-of-principal 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?

Sample sizes are determined based on previously published data, both within and outside our group, as well as the expected variation and effect sizes to ensure that experiments are performed with sufficient animals to detect biologically relevant differences.

From the scientific literature, we know there are likely to be sex differences in specific immune responses (e.g. role macrophage/microglia in pain mechanisms). Initial studies using a 50/50 split in males and females will therefore be tested for the influence of sex as a variable. Where an association with sex as an independent variable is found, additional studies with more animals will be performed to increase statistical power to test for sex differences specifically.

Our key data comparison will be between animals with ‘painful’ and ‘painless’ neuropathy, caused either by a focal nerve injury or immune modulation. Statistical calculations for behaviour tests, for example, will be performed based on the difference between these two outcomes in our preliminary data, as well as relevant reports in the literature.

A typical experiment involving nerve injury would use 16-24 mice in total, with a mixture of males and females distributed evenly between 2-4 groups depending on the experimental readout (e.g. behaviour testing or histochemistry).

These numbers per experiment have been extrapolated over the duration of the project based on the likely frequency of experimentation, the number of researchers working on the project, and the number of biological targets that can practically be investigated in this time.

Genetically altered mice required for our breeding programmes have been calculated based on experimental use, the expected frequency of progeny based on the known inheritance ratios, and the number required for breeding and colony maintenance.

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

The use of equal numbers of both sexes also increase our chance of observing sex differences, which are increasingly recognised as an important factor in neuropathic pain syndromes. When statistical analyses recording sex as an independent variable suggest possible differences, further experiments with more animals can be performed, thus restricting additional animal use to only when necessary.

The majority of animals will be bred on a C57BL/6 background. The C57BL/6 strain of mice has well- characterised behaviour and is commonly used for genetic alteration. Wild type offspring produced from the mating of genetically altered mice can be used as controls in experiments on genotype, or distributed to other lab members thereby reducing unnecessary wastage.

Animals will be randomly allocated to treatment group using a random assignment algorithm offered by the NC3R's Experimental Design Assistant. At the point of data capture and analysis the experimenter will be blind to genotype and/or treatment in order to minimise experimental bias.

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

Breeding colonies will be managed in line with the best practice guidelines. A mouse colony management system will be used to ensure efficient colony maintenance, including breeding regimen, and reduce the number of animals that we use.

This online system will assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced.

Cage and littermates of both sexes will be used in the same experiments, optimising usage of animals and animal husbandry.

Assessment of behaviour will be made in a dedicated, well-equipped quiet environment with which the animals have been familiarised, reducing variation and therefore reducing the number of animals needed per experiment.

Refinement

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

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

Focal nerve injury is anatomically defined. This method reduces the number of limbs involved in the neuropathy and provides the opportunity for the animal to both guard and withdraw the affected limb from an escapable painful stimuli, helping to avoid additional suffering, distress or persistent harm.

The sciatic nerve lies superficially in the hind limb, enabling a quick, targeted, and minimally invasive surgery to perform the nerve injury.

The sciatic nerve crush model is necessary to measure the relative proportion of axon integrity throughout the nerve and has long been used for studies of axonal degeneration and regeneration. This is the basis of the full and partial nerve crush comparison, where intact axons after partial crush generate a more neuropathic pain-like sensory phenotype than a full crush of the nerve in which all axons degenerate. Unlike full nerve transection, crush injury allows the regeneration of motor and sensory axons. Our recently published data show that motor co-ordination in mice with either full or partial crush returns to baseline within two weeks of injury. In addition, the sciatic nerve does not supply all of the muscles of the hind limb.

Therefore, mice are able to ambulate and feed as normal soon after surgery in the model. Comparing the partial versus full crush injury as models of ‘painful’ and ‘painless’ focal nerve injury, respectively, also represent an advance on the traditional 'injury versus sham' experimental paradigm. In this project we will be able to separate the specific immune factors driving neuropathic pain (present in the partial crush model) from the general immune response to tissue injury (present in both full and partial crush models).

Rodent models of neuropathic pain have traditionally involved damage (transection or ligation) of part of the sciatic nerve. This 'partial' neve injury appears crucial to the pathogenesis of chronic pain, as a full crush injury of the sciatic nerve does not result in the same pain-like behaviour. A mechanistic comparison between full and partial crush models is therefore a core aim of this project.

Mice will be tested for their response to touch, warmth and cool sensation on the affected limb (typically the hind paw) as well as the uninjured limb for comparison at various time-points after nerve injury. The threshold at which a mouse removes its paw will tell us about its level of sensitivity and the possibility the mouse is experiencing a pain-like sensation. This method ensures mice are never exposed to supra-threshold or intolerable stimuli.

Mice may also be placed in an innocuous chamber that they associate with previously administered pain relief. Preference for this chamber indicates whether an animal is experiencing ongoing, spontaneous pain. Even more simple tests in which mice are recorded (without intervention from the observer) as they explore an open environment, or elevated beam, which can tell us about anxiety levels in the mice. Measuring negative

affect in mice may be important as we understand more about the associations between mood and chronic pain in humans.

Mice will be video recorded in spontaneous behaviour assays, including place-preference, algogen (pain) and pruritogen (itch) assays to avoid the confound of having the experimenter in the room during testing. Place-preference is calculated via automated video tracking. Currently, algogen or pruritogen-induced responses are scored offline by an observer blind to the treatment group or genotype to avoid bias.

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

Non-mammalian animals are limited in their use because they either do not have the relevant type of immune cell or their immune system is too different from the human immune system to provide relevant results.

Alternative model organisms such as the Fruit Fly (Drosophila), Zebrafish and Roundworm (Nematode) demonstrate very different circuitry of the sensory nervous system compared to mammals. In some cases they lack key genes involved in sensation and which are conserved between rodent and human, and critically have a very different immune environment and therefore show very different functional responses compared to mammals.

We can't use very young animals as their response to nerve injury is not typical of that seen in adults (i.e. it usually recovers on its own) and the neuropathic pain-like behavioural response of very young animals is not well characterised. Focal nerve injury will not be carried out on animals below 6 weeks of age.

Neonatal and juvenile mice will be used however when gene transduction is required at an early stage of development. Subcutaneous administration of AAV9 viral vectors containing transgene induction sequences has been used successfully by colleagues to induce wide-spread neuronal expression within four weeks.

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

All surgical procedures will be performed with strict aseptic technique according to LASA guidelines with appropriate peri- and post-surgical analgesia. We will adopt a strict single-use needle policy for all injections.

When assessing behavioural outcomes in relation to sensory function we will assess the response to stimulation of a body part (typically the hind paw) with a natural stimuli, for instance a radiant heat source, from which the animal may escape by withdrawal of the limb. Animals will not be subjected to persistent, high intensity stimuli (such as pinch tests), which are by definition unavoidable for the duration of the test.

Individual touch and temperature stimuli will be applied only for the number of times necessary to accurately calculate the withdrawal threshold.

The frequency of behavioural testing will be reduced to that sufficient to reflect the ongoing sensory experience of the animal at key time points; typically daily in the first week after injury, reduced to weekly, then monthly testing. Only where absolutely required to answer

the question of chronic nerve injury will animals be kept beyond two months after surgery (up to a maximum of six months); this will be specifically to address the long-term persistent changes in the immune response beyond the immediate recovery from nerve tissue damage.

Animals kept for more than two months after surgery will undergo enhanced monitoring for the signs of aging. A checklist of the common signs of aging will be provided to researchers and animal unit staff.
The check list will contain information on the general action to be taken in the event of any organ- specific observation depending on the severity according to recently published guidance.

Pilot studies of new immune and neuroactive substances will first be carried out on smaller groups of animals. Dosing will be escalated with close monitoring of animals 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?

All experiments will be designed, performed and reported in accordance with the ARRIVE and LASA guidelines (https://www.lasa.co.uk/current_publications/), including guidelines for aseptic surgery. Experiments to assess behaviours indicative of a potential pain-like state will follow the PPRECISE guidelines.

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

The National Centre for the Replacement, Reduction and Refinement of Animals in Research (NC3Rs) website provides a comprehensive resource for protocols and the latest developments in the 3Rs in the UK.

All licensees will be encouraged to access the NC3Rs resources, and experiments will be designed and enacted using the NC3Rs Experimental Design Assistant.

Our institute also has an NC3R’s regional manager available for consultation, along with regular internal 3R’s meetings.

17. Mechanisms of embryonic heart specification and morphogenesis

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Developmental Biology, Cardiovascular development, Congenital heart diseases

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The project aims to decipher how the embryonic heart develops and how its development is sometimes disrupted, leading to congenital heart 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?

Congenital heart disease is the leading cause of infant death in the UK. It affects almost one live birth every 100; the incidence is even higher if foetuses that do not survive to term are included. Congenital heart diseases arise from abnormal heart development, so understanding how the heart forms during normal development is important.

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

The work will lead to an improved understanding of how the heart forms in the embryo during pregnancy. It will help us understand why birth defects occur and may help us find a way to prevent or intervene.

Data will be published in peer-reviewed open-access scientific journals and, where appropriate, in publicly available databases. It will also be disseminated at scientific meetings and in the broader press when necessary.

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

The work will improve a basic understanding of heart embryonic development and publications in peer- reviewed open-access scientific journals and publicly available databases. This will occur within a year or two of the licence award. Within three or four years, other scientists will begin to publish work informed by our studies, and in the longer term (say, ten years), we hope the work will help reduce developmental abnormalities in humans.

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

My research group and other research groups will use the outputs. When inferences from the work might be drawn about mechanisms of congenital heart diseases, clinicians will use the data. I have ready access to clinicians, whose objective is to translate basic research for human benefit as quickly as possible.

The outputs will become part of our fundamental knowledge of embryonic development and stem cell differentiation and will be incorporated into my university lecture course.

Others will also use the outputs to inform their experiments on embryonic development and stem cell differentiation. For example, our collaborators may wish to recapitulate the sequence of molecular events that occur within an embryo to make stem cells differentiate in a particular cardiac lineage.
Recapitulating the events during normal development may help produce stem cells that contribute to the proper cardiac tissues without harming the human patient.

Species and numbers of animals expected to be used

- Mice: 6000
- 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.

This project will investigate heart embryonic development. We propose to work on a mammal to enable the extrapolation of results to the human clinical situation. The mouse is the mammal of choice, owing to its small size and excellent availability of genetic tools,

which are critical for the proposed research. Significantly, mouse heart defects resemble that of humans. Genetic models of congenital heart disease are well-established in mice. These are available through transgenic lines already in-house or in public repositories.

We will also use a minimal number of surplus rats -when available- to extract rat serum to perform cultures of mouse embryos for 24 hr from embryonic day 8.5 in rat serum diluted with defined media. Rat serum usage cannot be avoided, to achieve high quality mouse embryo cultures, but rat usage can be reduced using medium containing diluted serum. Whole embryo culture is a valuable research method in mammalian developmental biology and birth defects research.

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

We will breed and maintain genetically altered animals up to 15 months of age.

We will administer substances such as transgene-inducing or deleting agents (e.g. tamoxifen and doxycycline), labelling agents (e.g. BrdU) or control substances of substances that modify the phenotype, alone or in combination or intermittently by an appropriate route and volume for their age (in diet or drinking water; orally by feeding from a pipette or by gavage or by injection (intraperitoneal).

We will produce rat serum by inducing and maintaining general anaesthesia using an agent appropriate for the species and route of administration and perform exsanguination via laparotomy with withdrawal of blood from a major vessel, or via thoracotomy with blood withdrawn from heart. Blood is centrifuged immediately to separate the serum and then prepared in the lab for use in embryo culture.

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

Breeding and maintenance of genetically altered animals: some of these Genetically modified lines may show an immediate post-natal lethality in 0-15% of newborns (unexpected death-before five days for mice – before ASRU does not require you to report any mortality). Surviving animals usually thrive and breed, as well as the background strain used. We will avoid breeding Het mice. Creating new mutants or crossing two lines with no/mild phenotype to screen for genetic interaction could produce a moderate severity.

Administering substance and embryo harvest: Occasionally, administering the transgene-inducing or deleting agent tamoxifen can lead to adverse effects such as termination of pregnancy or loss of appetite.

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

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

Mouse:
Mild: ~90%
Moderate: ~10 % Rat:
Non recovery: 100%

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 aims to understand how the mammalian heart forms during pregnancy. Heart development occurs within a pregnant female, so studying it without using an animal model is impossible. Heart development in the mouse is similar to humans.

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

In vitro systems such as mouse embryonic stem cells in culture (mESc) can capture certain early mammalian and heart development aspects.

Why were they not suitable?

A fully functional 3- Dimensional four-chamber heart does not form in in vitro systems.

The development of a better 3D in vitro system recapitulating the morphology of the developing heart during pregnancy is ongoing, and we hope, in, say, five years, to use the newly developed 3D in vitro system routinely in our laboratory to reduce and replace mouse usage. We also use mouse embryonic stem cells in 2D culture for projects analysing molecular pathways in depth. The proposed project is part of a larger project, which includes in vitro cell-based models of developments which feed into animal experiments.

Reduction

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

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

Our current funding runs out in May 2027. The total mouse numbers assume that our mouse usage will remain at current levels since our work to be accomplished requires the models, and, as yet, unknown experiments will be initiated as data is obtained and interpreted.

Mice are kept in “breeding” colonies of stable breeding pairs and “plugging” colonies used for experiments. Breeding mice need to be replaced when they reach~8 months old to maximise fecundity. The number of mice required for breeding colonies depends on the months the colony will need to be maintained.

The experiments require the generation of single, double and triple transgenic embryos. Preliminary studies estimate effect size and variance for power calculations. Collaborators in our department provide statistical advice to minimise the number of animals used for experiments. Pilot studies will first be conducted.

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

Lines not being used will be frozen.

In vitro and in silico computational work.

Use appropriate designs for quantitative analyses (i.e power calculations).

Ultimately, all results will be submitted for publication in peer-reviewed journals where anonymous referees will give an opinion on the reliability of the data and whether the conclusions can be satisfactorily drawn from the findings. Our journals of choice demand ARRIVE guidelines to describe datasets.

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

We strive to use and maintain low numbers of animals by sharing lines and tissues from genetically modified mice with others and freezing embryos and tissues 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.

GA animals have specific genetic alterations introduced spontaneously or via targeted genetic modification. These genetic alterations either introduce molecular reporters in the genome or alter the genome in specific ways, such as inactivating a gene in specific cell types. We use transgenic reporters for particular signalling pathways or gene activity and Reporter lines to visualise the labelling of specific cell lineages.

This project is focused on understanding embryonic heart development. For this reason, it is necessary to breed animals with these genetic alterations that affect embryonic development. Animals will be monitored daily, and any animals displaying distress, loss of appetite or inactivity will be killed.

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

It is impossible to use less sentient animals since they are genetically too distinct from humans. For example, the zebrafish has only two heart chambers, and the frog has three. Mammals have four heart chambers.

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

Animal suffering will be reduced to a minimum by appropriate training of staff. Animals will be housed in IVCs to protect their health status.

Due to the diversity and variability in the phenotype of GA mice, it is especially difficult to predict the phenotype of newly generated GA lines. In some cases, phenotypes of GA lines can cause moderate suffering, while in other lines, the phenotypes may be indistinguishable from wild-type mice. Mice will be monitored to assess the need for pain relief or an appropriate point to cull the animal by a schedule 1 method. Staff who deal with the animals daily have scientists' mobile numbers to expedite such communications and action. Any animal will be immediately humanely killed if it shows any signs of suffering greater than mild or transient or in any way compromises normal behaviour.

Mice may be administered transgene-inducing or deleting agents (e.g. tamoxifen and doxycycline), labelling agents (e.g. BrdU) or control substances of substances that modify the phenotype, alone or in combination or intermittently by an appropriate route and volume for their age.

Environmental enrichment will be given to the animals. We will avoid singly housed animals where possible. The mouse environment will contain enrichment such as fun tunnels, nesting materials and a chew block as toilet roll holders if housed singly. As a routine, mice will be kept in groups. Aggressive mice will be separated from submissive mice to minimise fighting. Non-recovery anaesthesia will be used to obtain rat serum. Appropriate volumes and numbers of injections will be used to minimise suffering.

In vitro cell lines systems will be used to refine which interventions are likely to be most fruitfully analysed in mice; for example, cells can be treated with different small molecules in multiwell dishes. The most efficacious compound would then proceed to in vivo testing.

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

PREPARE guidance, LASA and NC3Rs.

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

We will be informed of new methods trialled by teams using the facility. For instance, for robust genotyping protocols, faecal sampling reduces the invasiveness (if second sampling is required post ear clip, for example). We will no longer use tail tip for biopsy as it is not considered in line with PPL Standard Condition 4. Tamoxifen may be administered by pipette rather than IP injection or gavage (the latter may be required where induction is weak; therefore, animals would be wasted if gene recombination was too low).

18. Medical device assessment

Project duration

5 years 0 months

Project purpose

- Basis 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

Medical, Device, Regulatory, Non-recovery, Pig

Animal types	Life stages
Pigs	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 generate the data needed to facilitate the translation of novel medical devices into the clinical setting, such as the NHS.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 introduction of new medical devices for the treatment, diagnosis or monitoring of disease (e.g. stents, valves, catheters, shunts, robotic tools/instruments, imaging/monitoring devices and cameras) is critical to improving the treatments available to patients. In order for new medical devices to obtain approval for use in a clinical setting (e.g. the NHS) it is first necessary to generate the data required to demonstrate they are safe and effective. For many devices, this necessitates the use of a representative animal

model in order to meets the requirements of the regulatory bodies responsible for authorising their use (e.g. the UK's Medicines and Healthcare products Regulatory Agency (MHRA), the U.S. Food and Drug Agency (FDA) or the European Medicines Agency (EMA)).

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

Medical device safety and efficacy/performance data, generated to Good Laboratory Practice (GLP) standards.

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

In the immediate term, the data will be useful to engineers and scientist involved in the development of new medical devices. In the medium term (e.g. the first year following a study) it is to be expected that the data will be used by regulatory bodies, such as the UK's Medicines and Healthcare products Regulatory Agency (MHRA), the U.S. Food and Drug Agency (FDA) or the European Medicines Agency (EMA), when considering whether or not to grant approval for the device to be used in a clinical setting, such as the NHS. In the long term (e.g. 5 years after a study), it is to be expected that health care providers and patients will benefit as a result of the introduction of improved devices for the treatment, diagnosis or monitoring of disease.

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

The data generated in these studies will be to GLP standards to meet the requirement of the regulatory responsible body for approving the translation of the device into the clinical setting.

Species and numbers of animals expected to be used

- Pigs: 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.

Juvenile pigs will be used for these studies as anatomically and physiologically they share a close similarity to humans and they can be obtained at sizes covering the full range of human development.

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 trolly. Anaesthesia will be overseen by a specialist veterinary anaesthetist and will be induced while the animal is in the transport trolly by the injection of an anaesthetic agent. Anaesthesia will be 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 replicates 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. At the end of the study the animal will be killed, without regaining consciousness, by the administration of an anaesthetic overdose.

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

The animal will experience no more than mild transient pain and distress during the induction of anaesthesia as a result of being given an injection. Thereafter, the animal will be maitained at a surgical plane of anaesthesia, and therefore unable to feel pain, until it is 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)?

100% Non-recovery

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

- Killed

Replacement

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

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

Animals will only be used where it is absolutly essential to do so either to enable an assessment of a novel device to be undertaken to determine its suitability for full commercial development or where it is a requirements of a regulatory body, responsible for authorising the use of the device in a medical setting, to provide safety and efficacy data generated using a representative animal model.

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

It is to be expected that the suitability of the material and integrity of the device will be assessed using non-animal bench tests and that some performace data may be generated using studies conduced ex- vivo.

Why were they not suitable?

The purpose of the outlined work is to generate essential data that cannot be obtained using non animal alternatives. No device will be tested if a viable alternative to animal testing exists. By their very nature, medical devices are intended for use in living humans. Whilst tests to determine the suitability of the material and integrity of the device can be performed on the bench, for many devices it is not possible to fully assess safety and efficacy without the use of a representative animal model.

Furthermore, for most medical devices it is a requirement of the regulatory bodies, responsible for authorising the use of the device in a medical setting, that data demonstrating the safety and efficacy of the device is generated using a representative model, that is typically only possible using a live animal.

Reduction

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

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

The estimated number is based on enquiries to undertake related work. The number of animals needed for individual studies will be determined in consultation with the institute’s biostatistician.

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

Studies have to meet the requirements of the regulatory bodies responsible, which includes statistical analysis of the data generated. At the study planning stage, the establishment's biostatistician will be consulted to ensure that the study design is robust.

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

Where relevant, control data will be taken from related studies or obtained by the analysis of bio- banked 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.

Juvenile pigs will be used for these studies as anatomically and physiologically they share a close similarity to humans and can be readily obtained at a size suitable for the outlined work. All studies will be conducted under terminal anaesthesia. Consequently, animals will experience no more than mild transient pain and distress incurred by the insertion of a needle to induce anaesthesia. All other procedures will be undertaken while the animal is maintained at a surgical plane of anaesthesia. At the end of the study the animal will be killed without recovery.

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

In order to generate the data needed to meet the requirements of the regulatory bodies authorising the use of medical devices, it is necessary to use an animal model that is anatomically and physiologically similar to humans and of a size that enables the procedure to be undertaken in a manner that closely replicates that for which the device is intended. Medium sized mammals, such as the pig, are the only animals that meet the required criteria therefore, it is not possible to use a less sentient species for these studies.

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

Upon arrival in the unit the animals will be habituated to human contact by hand feeding treats. They will be trained to enter the weighing crate and transport trollies using food rewards. Anaesthesia will be induced by a specialist veterinary anaesthetist and maintained using mechanical ventilation. At the end of the procedure the animal will be killed without recovery.

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

Surgery will be conducted aseptically in accordance with LASA recommendations.

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

I actively participate in my institute’s 3Rs initiatives relevant to my work, developed through its AWERB and engagement with the NC3Rs including symposiums, workshops, meeting and NC3Rs prize competitions. I also stay abreast of 3Rs developments in my own research field by attending conferences and reading relevant scientific papers.

19. Next generation vaccines for livestock 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
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Sheep, Cattle, Vaccine, Vector, Infection

Animal types	Life stages
Cattle	juvenile, adult
Sheep	juvenile, adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To assess the efficacy of new vaccines for preventing infectious diseases of livestock.

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

Why is it important to undertake this work?

Infectious diseases of livestock are an important welfare issue for farmed animals and cause significant economic losses for farmers. Vaccination is a proven approach to prevent infection and reduce disease incidence but for many livestock diseases no vaccine exists or available vaccines are not effective. Prevention of disease through vaccination is

also simpler and less expensive than trying to treat or manage animals suffering from infection diseases. It is therefore important to research new ways of designing and using vaccines to maximise their potential in preventing disease. This project will evaluate viral vaccine vectors and virus-like particles as novel platforms for preventing infectious diseases in sheep and cattle.

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

Success during this project will identify new and more effective vaccines for use against diseases in livestock. Once proof of principle has been demonstrated, additional developmental work would follow towards commercialisation of these vaccines so that they can be sold to farmers and used to control diseases on farms.

We also expect to gain valuable new information on the relative efficacy of different vaccine designs in sheep and cattle. This new knowledge will result in peer-reviewed, open access, scientific papers and patent applications, and will be valuable information for other groups working to develop new vaccines for livestock.

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

The production of improved vaccines for sheep and cattle diseases will reduce disease incidence and improve animal welfare of farmed animals. Reduced disease will also improve the financial stability of sheep producers, with knock-on benefits for sustainability of rural communities. In the medium to long term, this research will therefore directly benefit vaccine manufacturers, the farming industry and other stakeholders in rural communities, In addition, the general public will benefit from increased food security and reduced environmental impact of farming associated with a reduction in animal disease.

Other groups benefiting from this research include scientists working on livestock vaccines in the UK and around the world.

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

If successful protection is obtained using these novel vaccines, we will seek to protect intellectual property by patenting. We will approach animal health companies and vaccine manufacturers to identify partners for commercialisation of the vaccines. We will engage directly with the Veterinary Medicines Directorate and the European Medicines Agency as appropriate to confirm the pathway for securing regulatory approval for the new vaccines. We have experience of commercialising livestock vaccines and have a strong network of existing collaborations with livestock producers and with companies specialising in animal health.

Research findings will be publicised and disseminated through publication and presentations at scientific meetings, such as the British Society for Immunology, the Microbiology Society, and the International Veterinary Vaccine Network. In addition, we will disseminate the information to members of the public, including livestock farmers and vets at agricultural shows and other livestock events. We have regular contact with colleagues in Scottish Government, ensuring that new knowledge arising from this research will reach policymakers.

Species and numbers of animals expected to be used

- Cattle: 150
- Sheep: 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.

The project aims to develop new vaccines for use in preventing infectious diseases of sheep and cattle. For this reason, we need to use those 2 species to assess the function of the vaccines being studied. Therefore, the experiments will be performed in the target species.

In general, vaccines for livestock are typically administered to young animals that are no longer protected by maternal antibodies. We will therefore typically use animals of 4-12 months of age in our studies. Where appropriate, we will also test for efficacy on the vaccines in older animals to understand if the vaccines perform differently with increasing age.

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

Sheep and cattle will be kept in standard farm accommodation for large animals, including stables with straw bedding. For infection studies involving high risk zoonotic pathogens, such as louping ill virus, they will be housed in high containment animal rooms for short periods (up to one month – including 7 days acclimatisation period).

Test vaccine preparations will be administered by injection using any standard route. Typically, this will be by injection into a muscle or under the skin, but if appropriate may be delivered orally, intravenously, or intranasally. The typical volume of each injection will be 1-2 ml. Animals may receive additional, booster, vaccines up to a further 3 times, typically at 3 week intervals, either by the same route or by a different standard route. From experience, animals will experience mild and transient discomfort from administration of vaccines. Blood samples will typically be collected on one or more occasions by jugular venupuncture before and/or after vaccination. The volume of blood taken at each bleed is within well accepted guidelines. E.g., for sheep and cattle on most occasions volumes taken will be 10 ml. If appropriate for the specific vaccine under study, intra-nasal secretions may be collected from sheep and cattle using a swab or brush as appropriate.

On some occasions, we will test the efficacy of a vaccine by injecting the animal with live pathogen (e.g., virus or bacteria). This is necessary to know if the vaccine can prevent or reduce the development of disease. In such experiments, we would expect non-vaccinated animals to suffer disease pathology. In addition, if a vaccine fails to protect against disease, we would also see disease in vaccinated animals. In such challenge studies, animals willl be monitored daily for evidence of the onset of signs of disease and scored using a clinical assessment matrix specific for the disease model. Animals will be euthanased according to specific humane end-points pre-determined for each model.

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

Administration of the novel vaccines would not be expected to have any lasting harmful effect on the animal. Past experience has shown animals exhibit transient discomfort upon injection and then recover quickly. There is a slight theoretical risk of localised bacterial skin infection following vaccination. Regular observation of the site of challenge will enable antibiotic treatment to be initiated promptly should this occur. In our previous studies, no antibiotic treatment has been instituted and no animal has ever been removed from a simple vaccination study on welfare grounds. Animals will be clinically monitored and deviations from normal will be treated appropriately by NVS or attending veterinarian. Where recovery is deemed unlikely, animals will be humanely killed by an approved Schedule 1 method.

The blood sampling procedure is also expected to cause transient discomfort but the animals should recover quickly (within 1 minute). The blood volumes taken should not have a negative impact on the animal's health.

In experiments where we test the efficacy of vaccines by challenge with infectious pathogen, animals may develop the early clinical signs typical of the disease under study. For example, we will administer louping ill virus to sheep by subcutaneous injection to test the efficacy of candidate vaccines. Based on our experience with this model (using 36 sheep in total), it is anticipated that in unvaccinated control sheep a fever will develop at or around the third day postinfection and temperature will return to normal by the eighth day post-infection before rising again in a second fever peaking at day 12. From around day 10 post-infection, a progression of neurological clinical signs may be evident, beginning with signs such as dullness and loss of condition (designated 'mild'), extending to include occasional or intermittent presentation of neurological signs (e.g., spontaneous nibbling, ataxia, head tremor, body tremor (designated 'moderate'). We will monitor the progression of these clinical signs and score against our clinical scoring matrix. Should any sheep display 'moderate' signs for three consecutive days that animal will be euthanised. If left unchecked louping ill infection can ultimately result in more advanced signs of neurological disease of increasing severity, including paralysis and convulsions (severe signs). Based on our experience with this infection model, we are confident we will be able to intervene before such severe signs become apparent.

As the project develops we may add additional protocols for additional disease models if we have encouraging results for specific vaccines. These will be added by amendment to this licence and we will assess the harms and potential adverse effects for those additional models on a case-by case basis when writing those amendments.

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

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

Cattle: 100% mild, 0% moderate, 0% severe
Sheep: 70% mild, 30% moderate, 0% severe

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?

We need to use animals to understand how immune responses are generated in response to vaccination. This process happens in lymphoid tissues that are very complex structures with many different cell types interacting and communicating with each other. In addition, these immune cells are in constant movement that allows them to interact with different partners at different stages of their development. These processes are so complex that currently no *in vitro* system is able to replicate this.

We also need to use animals to evaluate the ability of a test vaccine to protect against subsequent infection with the pathogen being targeted. Infection and the development of disease is a process unique to each pathogen, and involves a complex interplay between the pathogen and multiple cell types within the host. The range of host and pathogen factors that can be involved is not completely understood for any of the pathogens we will target, and so this is not possible to study without animals.

Should any of our vaccines successfully be developed for commercial production, we would be expected to have some evidence of protection in the host species for regulatory approval and licensing.

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

Non-animal derived alternatives including cell lines, organoids and cultured tissue sections have been identified and will be used whenever possible to generate data. In practice this means they will be used to fully characterise the viral vaccine vectors prior to their administration to animals. This ensures that the vaccines are optimised before being evaluated in live animals.

No alternatives for studying immune responses exist at this time.

Why were they not suitable?

Non-animal derived alternatives as listed above, while valuable for characterising the vaccine vectors, are not suitable for studying immune responses or for determining if the vaccines can protect an animal from infection. For that, we require the complex network of immune cells that only exists in the live animal.

Reduction

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

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

The numbers that will be used are based on planned funded experiments and experience doing similar studies in the past five years.

During the licence period, we anticipate testing at least 3 new cattle vaccines and 3 new sheep vaccines. Immunogenicity trials will involve small cohorts of animals in pilot studies. Vaccine candidates that show promise in the pilot studies will be tested in larger studies where vaccinated animals are challenged with the infectious agent (virus, bacteria, etc.).

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

We will test new vaccines in small pilot studies to determine if they are able to induce an immune response. This approach reduces the number of animals enrolled in vaccine trials by eliminating vaccine candidates that do not perform as intended before trials with large numbers of animals are initiated. Only vaccine candidates that perform as designed in the small pilot trials are carried forward.

We will consult with a biostatistician to ensure the number of animals in each study is appropriate based on the anticipated effect size. This will also take account of the results of pilots studies as well as the relevant literature on this topic for the pathogens investigated. The Experimental Design Assistant (https://www.nc3rs.org.uk/experimental-design-assistant-eda) will be used if a more complex design is needed.

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

Pilot studies will be done whenever no or limited data are available. In practice, this is likely to be the case for the majority of studies as it will be the first time the vaccines have been used 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.

For most of the work covered under this licence, we will vaccinate and give a boost vaccination to the target species and take blood samples to assess the immune response. In these cases there will be little or no suffering other than the mild and transient discomfort of a needle stick.

In the case of challenge studies with louping ill virus, we will administer louping ill virus to sheep by injection under the skin. We will then monitor the progression of clinical signs of louping ill over the following 2-3 weeks. From experience, we expect to observe a short fever followed by signs of neurological disease including nibbling, muscle tremors (facial and body) and dis-coordinated movement. Importantly, we would expect to observe such adverse signs only in unvaccinated control sheep and not in vaccinated sheep. Sheep will be scored daily according to our clinical scoring matrix for louping ill and we will intervene once these moderate signs have been present for a maximum of three days. This should prevent any sheep developing the severe effects of the model.

Other disease models may be added to this licence later by amendment. However, because it is unclear which of our test vaccines will deliver positive results in initial studies, we cannot include those at this point. The information provided for louping ill serves as a guide to the level of detail that will be provided.

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

Cattle and sheep are the target species for the pathogens we are developing vaccines against. Therefore, they are the most appropriate species in which to test the vaccines. In general, it is most appropriate to develop vaccines in the species they are intended to be used in.

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

The majority of the proposed animal work is limited to vaccination and blood sampling to assess the immune response. Thus, there will be little or no suffering other than the mild discomfort of a needle stick. We will continue to follow best practice as it develops regarding the use of adjuvants.

For animals involved in vaccine efficacy studies, each animal will be closely monitored and scored against a clinical scoring matrix that will be developed for each specific disease model used to define humane endpoints. A plan for managing adverse effects will be put in place for each pathogen and model. Pain management will be provided if needed. Additional species-specific refinements will be implemented where appropriate. For example, sheep will always be housed in groups of at least 2.

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

I will follow the NC3R guidelines (https://nc3rs.org.uk/the-3rs). LARN (Large Animal Research Network) publications/website

The ARRIVE (Animal Research: Reporting of In vivo Experiments) guidelines 2.0: Updated guidelines for reporting animal research. BMC Vet Res 16, 242 (2020). (https://doi.org/10.1186/s12917-020- 02451-y)

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

I regularly inform myself by reading relevant literature on 3Rs (mostly online). For example, since attending the FRAME training course on experimental design in 2020, I have received monthly newsletters containing information on activities and links to useful articles online. I will also contact others, including local AWERB members, the NVS and the local establishment 3Rs committee, prior to beginning any experimental work to ensure the planned work is appropriate. I also routinely search databases on published information for studies I am intending to avoid possible duplications.

20. Studying the biology of brain tumours

Project duration

5 years 0 months

Project purpose

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

Key words

Cancer, Brain, treatment, inflammation, immunotherapy

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to improve our understanding of the pathways and processes that promote the development, growth and pathology of brain tumours.

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

Why is it important to undertake this work?

It is important to undertake this work as different types of brain tumours cause significant illness and death in people throughout the world. Glioblastoma is the most common type of malignant brain tumour and is responsible for more deaths in people under forty years of age than any other cancer. Meningiomas are responsible for 1/3 of all brain tumours and cause significant morbidity, such as impaired coordination, altered behaviour and social

problems in sufferers, and can be fatal. Vestibular schwannoma tumours are highly linked with the NF2-related Schwannomatosis syndrome, and are life- shortening and life-limiting tumours, with substantial morbidity including hearing loss. There are currently no effective treatments for these tumour types, principally due to the lack of understanding of the biology of the tumours. Animal models provide critical opportunities to identify and mechanistically test the processes and pathways responsible for promoting the development, growth and spread, and pathology, of brain tumours, which will directly inform the identification of new treatments for the conditions.

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

The work in this project will lead to significant new information on the pathways and processes that promote brain tumour development and related suffering and mortality. Direct outputs from the work will be peer-reviewed research articles, dataset resources that will be shared with the research community, and presentations, where we will disseminate our discoveries.

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

The outlined programme of work will provide essential insight into the most appropriate molecules and pathways to target as new treatments for the different types of brain tumour. In the short term, this will be of major benefit to researchers and clinical scientists working on human brain tumours, increasing our understanding of the diseases. In the mid-term, the work should provide the foundation for clinical trials of new therapies for brain tumours, which, in the long-term, will ultimately help improve the standard of care treatment for the different brain tumours, substantially reducing the morbidity and mortality of people with the diseases.

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

We will publish our results in peer-reviewed journals, in open-access format. We will also initiate new and build upon existing collaborations to enhance the impact of our results. We will disseminate unsuccessful approaches or negative data through specific journals or online forums.

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.

Mice are the most appropriate species for this work as murine brain tumour models are the most well characterised of the various animal models (when using established tumour lines, as will be done in most experiments within this licence). There is a significant body of literature that results obtained in murine brain tumour studies are relevant for

understanding human brain tumours. Murine models (using adult animals) are also highly informative for studying the efficacy of new treatments for brain tumours.

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

The general project plan will involve either utilising genetically modified animals that are predisposed towards development of brain tumours, or the implantation of brain tumour cells into targeted areas of the cranium or body by surgical routes. These approaches will be utilised to study different types of brain tumours with different locations, biology and severities. The models studied will involve benign vestibular schwannoma brain tumours, which in humans develop associated with the ear canal, meningioma tumours, which develop on the outer membranes of the brain, and malignant glioblastoma tumours, which form within the brain tissue. Experiments will utilise both male and female mice, to avoid experimental bias and to assess any sex-related effects on brain tumour biology.

Depending on the question addressed in each experiment, mice may receive injections to modulate the immune system or physiological processes, or may undergo surgery to modify tissue function (e.g. ligation of brain lymphatic vessels to change how cells and molecules drain out of the brain), or to enable visualisation of the brain tumour (e.g. removal of parts of the skull for microscopy analysis).

They may be administered chemotherapeutic drugs, or undergo irradiation with bone marrow reconstitution to change the nature of immune cells. Injections can be by different routes depending on the research question and the nature of reagents administered (i.e. reagents may be injected directly into the brain or cerebrospinal fluid, or provided systemically into the blood). The vast majority of animals will receive less than 4 injections to manipulate the immune system or physiological processes, by a maximum of two different routes. Some experiments may be short duration of 7 -14 days (when assessing the biology of fast growing and malignant tumours or when studying the very early phases or brain tumour development); however some experiments may be > 60 days, when studying the development of slow growing or benign brain tumour models. Multiple manipulations in a single animal will be avoided, when possible. Cumulative effects (e.g. additive effects) of multiple treatments will be minimised by allowing animals to fully recover from procedures such as surgery or irradiation and reconstitution before the animals undergo any subsequent treatments.

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

The different brain tumour models have the potential, related to their growth and effects on brain and nerve activities, to cause suffering. The models to be employed in the objectives are all well established and standard in the field. This ensures there is substantial information describing the trajectories of brain tumour development, supporting definition of the observable signs of suffering to appropriately determine time points of analyses in experiments, and to specify stop-go points and humane end points.

The suffering of animals with brain tumours will be minimised by closely monitoring all animals using a well-defined 7-point grading system assessing neurological activity (behaviour abnormalities such as tremors or circling), general signs of suffering (including hunching, piloerection, ataxia and lethargy) and monitoring weight loss (which is a very sensitive measure of animal health during brain tumour studies). Robust humane

endpoints for weight loss (20%) and level of adverse effects (balance, behaviour) will prevent unnecessary levels of suffering. Most of the modulatory procedures performed, or the reagents administered, to manipulate brain tumour development or growth should not directly promote animal suffering. Analgesia will be given to reduce pain following specific protocols (such as following surgery to ligate lymphatic vessels or create a cranial window); however, the direct damage caused to the brain by the brain tumour lessens analgesia efficacy in that context. Moreover, brain tumours do not cause pain or suffering for substantial periods of time following development, and when the brain tumour begins to cause pain (identified using our clinical grading scale), the experiment will be terminated. All administrations will be performed via the most appropriate route through (when applicable) the careful control of injections.

Expected severity categories and the 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 total, on this licence, 37% of animals are expected to experience mild suffering, 23% of animals are expected to experience moderate suffering, and 40% (genetically altered animals bred within protocol 1 and control genetically altered animals within the brain tumour protocols) will experience sub- threshold suffering.

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 can only address the majority of our questions when a complete immune system and central nervous system, as well as auditory system, are present in their normal anatomical and physiological configurations (for example where the brain tumour can connect with draining lymph nodes to establish anti-tumour responses, when peripheral cells can migrate to and enter tumours, and when brain tumours can influence nerve function and hearing), or when the brain tumour is in a clinically relevant environment (for example where the brain tumour is acted upon by the various cellular, circulating and structural components within the cranium): the use of animals is, to a significant extent, unavoidable in our experiments.

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

When we have simple and reductionist questions, such as how brain tumour cells directly interact with specific immune cells or brain cells (i.e. nerve cells), or how they respond to specific molecules, we will establish *in vitro* co-culture systems (culturing more than one

type of cell together, such as brain tumour cells with immune cells) to study these defined interactions. These *in vitro* systems will also be utilised as a strategy to test and prioritise treatment and modulatory approaches for subsequent employment *in vivo* within animals, to examine the pathways that influence brain tumour growth and pathology.

Why were they not suitable?

Such *in vitro* co-culture approaches are suitable for only very specific questions as during the course of brain tumour development, growth and spread *in vivo* within animals, the interaction between cancer cells, immune cells and brain cells is often shaped by a myriad of factors, including circulating immune cells and immunological mediators (produced in the brain but also recruited from other parts of the body), and the multifaceted communication with other brain resident cells. Thus, for the majority of our investigations to obtain accurate and clinically relevant results, we need to study our objectives within intact physiological tissues, *in vivo* within animals or *ex 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 numbers of animals based upon our previous experience of running similar project licences in the last 15 years, as well as from consulting relevant literature and engaging with collaborators employing similar models. Thus we have accounted for the nature and requirement of the projects we have immediate plans to perform, including the numbers of times experiments must be repeated, the numbers of different experimental groups in experiments, and the numbers of mice required in different groups. We have also estimated the number of animals to be used based upon future plans and collaborations.

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

We calculate the required group size using data from previous experience, and published work. This ensures that we have sufficient ability to detect a biologically relevant effect using as few animals as possible. We also perform sample size calculations based upon pilot and preliminary experiments to ensure we perform subsequent experiments with the correct number of mice to detect statistically significant results. We also adhere to ARRIVE and PREPARE guidelines for reporting of research involving animals, which outlines appropriate study design (e.g. control groups and sample sizes), how to avoid experimental bias, and the analytical framework for simple and complex 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 perform pilot experiments when undertaking new experimental approaches so we can discontinue uninformative or inappropriate methodologies and so we can also evaluate the variability and magnitude of experimental effects. This will allow us to accurately assess the numbers of mice to use in future studies. We also consult the literature when we are performing similar approaches as others have previously performed, in brain tumours or in other models. This allows us to predict the strength of expected effects within our experiments, and therefore, the numbers of mice that need to be used to detect statistically and biologically relevant results. We will carefully manage maintained colonies (i.e. by employing short-term harem breeding) to ensure we have sufficient numbers of mice for planned experiments but ensuring we do not have surplus mice. Any unneeded mice will be shared with researchers, who have authority to receive 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.

Mice are the most appropriate species for this work as murine brain tumour models are the most well characterised of the various animal models (when using established brain tumour lines or genetically engineered models, as will be done in most experiments within this licence), giving us essential background information that is lacking in other systems.

Mice are also the animals of choice for immunological investigations as so much is known about their immune systems, different well-characterised inbred strains of mice exist with differing responses to disease, there are a large number of genetically modified murine strains available for use, and all the reagents that we require (such as for modulation of the immune system) are available.

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

To obtain informative results in this project we need to utilise an organism with a complete immune system and physiologically equivalent brain and nervous system to that in humans, in which the different types of brain tumours can be established. This precludes the use of less sentient drosophila models. Whilst zebrafish models for Glioblastoma exist, these are less established for Meningioma and Vestibular Schwannoma. Challenges still exist for accurate and reproducible establishment of orthotopic xenograft Glioblastoma models (which is the injection of human-derived brain tumour cell lines into the Zebrafish brain). All technologies and methodologies required for addressing the project objectives are established in mice, and are not easily transferrable to Zebrafish.

We must also use adult mice with a fully formed and functional immune system. Otherwise, our results would be difficult to translate to the study of human brain tumours. We will

perform certain protocols under terminal anaesthesia but due to the length and course of experimental brain tumour models, it is not possible to perform all work under anaesthesia.

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

Due to the overall purpose of this work - to study the factors responsible for development of and treatment response of brain tumours - we do need to allow experiments to proceed to the point where animals will experience some suffering, recapitulating the development of symptomatic brain tumours in humans. However, in our experiments we do not require animals to progress to experience severe suffering (i.e. experiments will be terminated before brain tumours cause severe suffering). Moreover, animal suffering will be minimised by providing analgesia, when possible and when required. For example, whilst we can provide analgesia following surgery, we are unable to provide analgesia to mitigate the effects of the brain tumour, as the analgesia itself will modify animal behaviour and the course of the experiment. Multiple treatments to manipulate the immune system or physiological process within a single animal will be avoided, when possible, with a maximum of two separate approaches applied in any animal.

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

We will follow NC3Rs guidance and we will continually assess our experimental designs in relation to advances within the relevant brain tumour and immunology literature.

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

We are committed to identifying and implementing new advances relevant for our work. We survey the literature for new refinements and experimental approaches. We actively engage with our institution (as well as receiving updates by email) to improve best practice in experiments. We are also on the NC3Rs mailing list to obtain newsletters with new advice, guidelines and information. Standard operating procedures for users working with animals are in place within our institution, the adherence to which is compulsory, which incorporates advances in animal handling and ensures animal welfare.

21. Super High Affinity Sheep Antibody Creation

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)
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Sheep, Monoclonal, Antibody, Diagnostic, Monitoring

Animal types	Life stages
Sheep	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To provide a reliable and effective service to produce high sensitivity sheep monoclonal antibodies (SMAs) and supply to external research groups and/or commercial clients for clinical diagnostic testing/food safety testing/environmental monitoring purposes.

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

Why is it important to undertake this work?

There are unmet needs when it comes to affordable clinical diagnostics in a number of diseases/conditions, a notable example being Alzheimer's disease/dementia. In this case,

current diagnosis requires expensive hospital scans and is often not carried out until significant cognitive decline has already taken place, harming the chance of a positive intervention. In this Project Licence, through collaboration with academic partners, our technology could facilitate affordable, simple blood tests that could be used to identify the signs of this disease earlier in its progression, thus allowing a better patient outcome.

Environmental monitoring is a growing area of concern for governments and industry who want to better understand the impacts of pollutants on those they are responsible for, be that employees or the wider population. Again, our technology utilised in this PPL could lead to simple tests that are able to assess the level of an individuals' exposure to a pollutant and/or human impact on the environment around us.

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

Under previous similar PPLs, the establishment has successfully created SMAs to various markers of ill health, including cancer, heart disease and dementia. Through our commercial partners, these SMAs have facilitated routine clinical tests that have improved patient outcome around the globe, and through academic partners furthered our understanding of disease.

During the course of this programme of work, we intend to add to our back catalogue of successful projects with a new panel of SMAs to a range of clinical diagnostic/food safety/environmental monitoring targets. These SMAs will target either a known marker in order to facilitate an improvement to an existing test, or a new marker entirely thereby facilitating a completely new test. Given our track record, we have a very high expectation of success in this endeavour.

Whilst we do not publish our work directly, we anticipate some of these SMAs to be used by academics in publications, as has been the case under previous PPLs.

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

Our SMAs have been proven to give rise to more sensitive & accurate diagnostic tests, enabling clinicians to better diagnose, monitor & treat human disease and ill health. With our commercial collaborators, typically we supply SMAs that they develop into a test for use on one of their devices, a process which can take 2-5 years to complete. The benefit of such SMAs made under this PPL will likely not be realised within the lifespan of the licence, but clearly they provide a long term benefit.

Whilst our overriding aim when collaborating with an academic institution is typically to bring a new diagnostic marker/test to market – a process which will likely take at least 5-10 years and is therefore a very long term benefit – by freely sharing the SMAs we develop with our academic partners we hope to be able to facilitate the fundamental understanding of how a disease state progresses in key areas of human health (e.g. heart attack, dementia, etc.). This is therefore a short to medium term goal that could happen within the lifespan of this licence.

Many diagnostic tests still use polyclonal antibodies (derived from repeated immunisation & blood sampling of animals) which require constant use of animals over time. Monoclonal antibodies (derived from animal cells grown in the laboratory) only require animals to be used once during their production. Therefore, by replacing the polyclonal antibody element

of a diagnostic test with a monoclonal antibody, fewer animals will be used in total.

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

The establishment actively seeks collaborators whenever embarking on a new project, be that a new or existing commercial partner, academic partner, industrial contact or regulatory authority. Whilst we rely on these partners to market any resulting test and/or submit publications using our SMAs, we provide ongoing support in both cases through regular communication and foster positive working relationships to maximise our chance of success.

Species and numbers of animals expected to be used

- Sheep: 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.

Large mammals have a larger diversity of white blood cells than rodents (mice are most commonly used for antibody production). These cells produce a broader range of antibodies including examples with exquisite specificity and sensitivity uncommon to smaller mammals. Of large animals, sheep are suitable for several reasons:

Ease of immunisation and blood sampling procedures – Sheep usually remain placid while being held. Whilst they do not like to be singled out, they remain calm as long as they are within sight of other sheep and are in no visible pain. Procedures are completed very quickly (usually within a couple of minutes), so any stress of being restrained is transient.

Ease of housing – can live a “natural” life on a farm.

Availability of a cell line in our laboratory that allows us to make the sheep’s white blood cells (which produce the antibody) “immortal”. This is the cornerstone of our technology.

We propose to use adult sheep between 0.5-4 years old, when they are in their physical prime. Younger than this could cause unnecessary stress to lambs and they may not have as fully developed an immune system which could lead to unsuccessful immunisations resulting in more animals being used. Older animals could be more vulnerable to stress and age-related health complaints. Sheep will typically be 0.5-2 years old at the outset of our protocol but so as not to waste animals, sheep may enter this protocol at any time until age 4 years.

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

All sheep are housed as a flock on outdoor, natural pasture with plenty of grass and a source of clean water. When necessary supplementary feed is provided, for example during winter months.

An animal will undergo 3-9 (typically 4) immunisations at a minimum of 3 week intervals (typically 4 weeks). These each involve injection of a small volume (~0.25mL) of non-harmful liquid containing our antibody target under the skin at 4 sites on the sheep's body (left & right leg, and left & right shoulder areas). Once before starting immunisations and then 1 week after each subsequent dose, a small vial of blood (~10mL) will be taken from the jugular vein of each sheep using a needle and vacutainer system. These blood samples will be compared in the laboratory to assess the success of the immunisation regime. A typical immunisation course will last ~3 months. At the end of an immunisation course, all sheep will be euthanised humanely by a veterinarian using a Home Office approved method.

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

A relatively low number of animals experience localised inflammation/abscessation at the immunisation site as a result of injection but do not display any signs of discomfort or ill health.

Expected severity categories and the 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 methods used are classified as “mild” severity by Home Office classifications.

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 establishment's unique technology is the use of a specialised type of biological cell that when fused with an antibody-producing cell from a sheep gives rise to an "immortal" cell line that will continue to grow and produce antibody indefinitely. Without this "fusion partner" an antibody-producing cell cannot be kept alive indefinitely.

Diagnostic tests that rely on antibodies (immunoassays) utilise the exquisite specificity of antibodies for their target, i.e. they will bind to their target and nothing else. The stronger the binding of the antibody for its target (affinity), the more sensitive the diagnostic test will be.

For any given target, we therefore first need cells producing antibodies that bind to that target. Generating these antibody-producing cells relies on challenging the immune system

of a live animal in order to create the diversity necessary to obtain the one cell producing the antibody of interest (i.e. the most specific and/or most sensitive antibody to that target). The complexity of immune system and therefore the diversity of antibody-producing cell generated by a live animal, cannot be adequately replicated by non-animal means.

Sheep are known to produce both high affinity antibodies and a high diversity of antibody-producing cells, so are ideal for this use.

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

Non-animal based antibody/binding entity production technologies, such as DNA library phage display and aptamers.

Why were they not suitable?

Non-animal alternatives such as display technologies or aptamers have existed for many years but do not routinely produce antibodies/binding entities that are able to match the level of affinity of a sheep monoclonal antibody.

Before undertaking any immunisation protocol, the establishment always attempts to source available third party antibodies to any given target which can be derived from either animal or non-animal means (although this is not possible for many of the novel targets we work on). Whilst these third party antibodies can prove useful for validating the conformation/structure/immunogenicity of a potential antigen, they never have the level of affinity required by our customers/collaborators, who will have likely already tested them and found them unsuitable, since if they were suitable they would provide a much more cost-effective solution than our service.

In our experience, such antibodies are at least 10-fold lower affinity than SMAs. Since affinity is an intrinsic characteristic of an antibody, there is only so far this can be compensated for by intelligent assay development/technology and a lower affinity antibody will always result in a lower sensitivity immunoassay. They are therefore not a suitable alternative to SMAs in this programme of work but can be used as an adjunct in certain situations (see Reduction section).

Reduction

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

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

It is estimated that less than 100 animals will undergo procedures annually. This is based on a realistic maximum of 20 antigens per year; 5 animals per antigen. Under similar licences in 2004-2022 inclusive, a total of 777 animals were used (40.9 per year average) with a maximum of 65 used in one year. We expect our level of animal usage to remain similar or be slightly higher than average during the course of this project licence.

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

Establishment staff have extensive experience of judging the likelihood of a given immunogen (the substance administered to the sheep) provoking the desired immune response, and use this to ensure effective immunogen design. The establishment has longstanding relationships with a number of contractors who commonly prepare immunogens to our specific design – a process which has shown repeated success throughout previous PPLs. Where possible, immunogens are tested to validate their fidelity of structure/sequence prior to immunisation. These factors minimise the risk of wasted animals, thereby reducing the overall number of animals used.

Our historical data shows that there is high variability in immune response amongst animals immunised with the same immunogen, therefore using more than one animal per group of antigens is essential to obtain the desired antibody. In-house immune response data is constantly monitored to ensure the number of animals per antigen is sufficient but not excessive.

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

For certain targets it is desirable to improve sensitivity and specificity by making an antibody that recognises an immune complex (i.e. another antibody already bound to its target). In these instances, the affinity of the antibody within the immune complex is most important and therefore would be an SMA. The anti-complex antibody may not need as high an affinity and therefore could be made via a non-animal method (e.g. in vitro display), reducing the number of animals used.

The establishment has a strong commercial relationship with a provider of this technology and has used them successfully in the past to provide just such anti-complex antibodies, which are in the process of being commercialised into products. We plan to expand our use of these methods in the future which will lead to a further reduction in the number of animals used.

We have considered challenging animals with multiple antigens sequentially in order to reduce overall animal use, but have ruled out this approach since the mainstay of the immune response comes from the primary immunisation given in combination with Freund's complete adjuvant (FCA), and to keep the severity of the procedure as mild as possible we only administer FCA once to any given animal. If we were to immunise an animal that had already undergone the immunisation protocol with a different antigen, we would not use FCA and therefore the potency of its immune response to the new antigen would be significantly diminished, leading to a reduction in antigen-specific lymphocytes harvested and therefore a much reduced chance of project success, ultimately resulting in additional stress to the individual animal and additional animals being subsequently immunised.

Refinement

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

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

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

No animal models of human disease will be used in this PPL.

Sheep will be immunised infrequently with small volumes of non-harmful immunogens and be subject to infrequent blood sampling. Both of these processes cause minimal pain/suffering and will be undertaken with other sheep present, minimising the stress to the animal involved.

All sheep will live a "natural" life in a field, be subject to good sheep husbandry practices including regular veterinary inspections, and given any necessary medications for health complaints with no restrictions.

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

Sheep are the only species compatible with the establishment's technology, so others cannot be considered.

Animals at a more immature life stage (foetal) would be impractical, be far more stressful and dangerous for the mother and not give rise to useful antibodies due to an undeveloped immune system, so they are clearly unsuitable for this purpose.
Production of antibody-producing cells within an animal relies on development of an immune response over time (several weeks) so using terminally anaesthetised animals would be unsuitable. Also, the act of anaesthetising a sheep is likely more stressful than the immunisation and blood sampling procedures themselves.

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

Establishment laboratory staff have extensive experience in analysing test bleeds and using them to characterise the immune response of a given sheep. Taking these at regular intervals allows trends to be established which allow us to determine whether additional immunisations (and therefore additional stress) are necessary or we can end the immunisation protocol at that point.

In work done under previous PPLs, refinements to the precise location of the immunisation site and the technique used have minimised incidence of localized inflammation/abscessation but we will continue to monitor this and investigate any method to reduce this still further.

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

The resource library on the NC3Rs website contains specific guidance for replacing animal-derived antibodies with animal free affinity agents such as aptamers and molecularly imprinted polymers. As discussed, these are not currently of high enough

affinity or reproducibility to replace SMAs, but advancements in these technologies will be monitored for opportunities to further refine our process.

Whilst no published best practice guidance exists that specifically deals with refinement of immunisation of sheep for scientific purposes, there are relevant resources that cover vaccination of livestock as part of farm management. The Agriculture and Horticulture Development Board provides best practice advice on administering vaccines (ahdb.org.uk) that includes subcutaneous (beneath the skin) injection, which is the same route as our immunisation protocol.

We will also continue to refer to the Home Office published Guidance on the Operation of the Animals (Scientific Procedures) Act 1986, as well as the additional related documents available on the gov.uk website. In particular, AWERB members will look out for updates to these documents and circulate to others as appropriate.

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

Establishment staff receive updates from NC3Rs via their newsletter, will attend webinars and utilise resources on NC3Rs.org.uk where appropriate.
Any staff member is encouraged to raise opportunities to improve our work with respect to the 3Rs with the AWERB at our regular weekly meeting, where implementation strategy can also be discussed.

Establishment staff stay informed about the latest advances in antibody production technology, including non-animal methods by subscriptions to relevant scientific literature, attending appropriate conferences (e.g. AACC Annual Scientific Meeting), and by regular contact with customers and collaborators. We will look to apply these methods to our process where appropriate, such as has already been the case with our use of anti-complex antibodies (see Reduction section).

22. Synaptic and neuronal compensation in Alzheimer's disease

Project duration

5 years 0 months

Project purpose

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

Key words

Alzheimer's disease, synapses, dementia, neuroplasticity, compensation

Animal types	Life stages
Mice	juvenile, adult, 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?

Our aim is to deepen the understanding of the synaptic and neuronal compensatory mechanisms counteracting the progression of Alzheimer's disease, with the hope of developing alternative therapeutics to delay or even prevent the onset of clinical 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?

Alzheimer's disease remains an incurable disease and therapeutics targeting the

underlying mechanisms (e.g. amyloid plaque accumulation) have consistently failed in numerous clinical trials. These disappointing outcomes have highlighted the need to find alternative strategies to counteract the disease. By focusing on the protective mechanisms of the brain that deal with pathology, rather than the mechanisms which lead to pathology, this work has the potential to uncover novel avenues for the therapeutic development.

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

We will generate knowledge on how the brain cope with the loss of neuronal connections and ultimately, the loss of neurons at the early and later stages of Alzheimer's disease, respectively. Given that the mechanisms underlying resilience to Alzheimer's disease are almost completed unknown, the knowledge generated in the project will not only lead to original publications but more importantly, to the potential development of novel therapeutics strategies for Alzheimer's disease.

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

In the short-term, the knowledge generated in this project will benefit other researchers studying how the brain cope with the neuronal damage in Alzheimer's disease or those investigating alternative approaches to halt the progression of the disease. As this work has the potential of identifying molecules that can be targeted therapeutically in order to boost these protective mechanisms and delay the onset of dementia, this project will benefits the pharmaceutical industry searching for alternative therapeutical approaches to cure Alzheimer's disease. Ultimately, this project has the potential of benefiting individual suffering from Alzheimer's disease, who currently lack a cure.

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

We will attempt to maximise outcomes of our research through collaborations as well as presentations in international conferences. In addition, we are committed to open science and to the immediate, unrestricted online access to all of our published research findings. First, all our preprint manuscripts will be first made freely available to access and download on preprint services such as biorxiv.org.

Second, final peer-reviewed research manuscripts will also be made freely available to access and download immediately on publication, either via a publishing platform or journal route. Finally, we will share all our final research data and computer code in the appropriates freely available data repositories, upon publication.

Species and numbers of animals expected to be used

- Mice: 4500

Predicted harms

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

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

We will use mice due to the due to the availability of well-characterised transgenic mouse model of Alzheimer's Disease (e.g. APP/PS1) that recapitulates important aspects of the human pathology at the biochemical, cellular and behavioural level and are essential to test therapeutic interventions. In addition, the existence of brain atlases for mouse will facilitate the location of brain regions for genetic manipulations (e.g. virus injection).

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

Animals used in this project will undergo surgery, injections of substances, cranial implants, culling under anaesthesia and behavioural assessments. Experimental durations will range from acute (1 day) to several weeks.

Typically, we will use an in-vivo microscopy approach to visualise neurons inside the brain of living animal models of Alzheimer's disease. To that end, we normally remove a small portion of the skull (craniotomy) under anaesthesia and inject a small volume of a virus (Adeno-associated virus) carrying fluorescent markers directly into the brain. Immediately after virus injection, we close the craniotomy using a glass coverslip to create a "cranial window" which allow us to visualise fluorescent neurons using a microscope. Following 4-weeks after surgery to allow for animal recovery and the expression of the fluorescent markers, animals are placed under the microscope to visualise the structure and function of neurons using either a bench-top microscope or a "wearable" miniature microscope (<2gr) while performing a behavioral task. To understand the mechanisms underlying resilience to Alzheimer's disease, these protocols may be performed in conjunction with the administration of drugs and substances either directly into the brain drugs and systemically (e.g. intraperitoneally).

Given that we will monitor the emergence of neuronal compensation over time following the loss of neuronal connections - or neurons themselves- we will longitudinally image the same neurons in the same animals in a daily or weekly basis for up to a 1 month period. To evaluate the impact of neuronal compensation in the cognitive state of the animal, a range of cognitive tasks may be performed in parallel or in conjunction to the imaging.

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

Regarding the transgenic mouse models of Alzheimer's Disease used in this project, we anticipate that most of them will develep dementia-related symptoms - such as memory and cognitive deficits - required to understand what causes these changes and if drug/genetic interventions can reverse them. One transgenic line in particular, the hTau. P301S, may experience apathy, reduced food intake and social and motor impairments, in addition to the cognitive deficits. To minimise the harms associated with these symptoms, we have specified general humane end-points based on the animal apperance, posture and behaviour.

As this project involves brain surgeries in wild-type and animals models of Alzheimer's disease, the surgical procedure may result in adverse effects during anaesthesia such as mild respiratory and cardiac depression, mild hypothermia and dehydratation, as well as eye drying. Postsurgery, animal will experience pain and related discomfort, and very rarely, infections. To control and ameliorate these adverse effects, we will carefully monitor -and adjust if necessary - the level of anaesthesia to prevent respiratory and cardiac issues, use a heating pad to maintain body temperature, apply ophtalmic ointment to

protect eyes, administer saline to prevent dehydratation, and importantly, administer analgesics post-surgery and provide antibiotic treatment in case of an infection, as adviced by the veterinarian.

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

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

Severities are considered to be moderate (90%) and mild (10%) for all procedures. For survival surgical procedures, death from anaesthesia (<2%) and surgical complications (<5%) may occur. The APP/PS1 may develop seizures over time, where the animal may recover with minor signs or become rapidly unconscious and then does not recover consciousness at any point before death (<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?

Given the complexity of the brain, it is still impossible to recapitulate its function in cell cultures or neuronal modelling. This become particularly relevant when studying cognitive dysfunction such as memory loss that requires a behavioural component to evaluate the expression of a given memory. In addition, the longitudinal nature of studying age-related disorders such as Alzheimer’s disease make necessary to utilised ageing living animals.

Our research programme rests on the notion that compensatory mechanisms – at the synaptic and neuronal level - delay the onset of cognitive decline in Alzheimer’s disease. As such, we will implement in-vivo models of compensation instead of short-lived in-vitro preparations that might not capture long- term compensatory events.

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

In this project, we also considered the use of in-vitro neuronal cultures and computational modelling alongside animal experiments to enhance our understanding of neuronal compensation. Specifically, we will utilise in-vitro organotypic brain slice cultures, which are not subject to regulatory constraints, to investigate the cellular-level mechanisms underlying neuronal compensation under tightly controlled experimental conditions. After gaining detailed knowledge of the signalling mechanisms involved in neuronal compensation through these in-vitro studies, we will use animal models to validate these findings in-vivo as proposed here.

In addition, we aim to collaborate with experts in computational modelling to develop mathematical or computational representations of these mechanisms. These

representations will be essential in generating hypotheses that can be subsequently tested experimentally, first in-vitro and then in-vivo.

However, since Alzheimer's disease primarily affects cognitive functions such as learning and memory, we will ultimately assess the functional relevance of these in-vitro mechanisms and computational models by evaluating their impact on a range of cognitive tasks using animal models of Alzheimer's disease.

Why were they not suitable?

Neuronal cultures and computational modelling fail to recapitulate complex brain function such as memory formation and loss that are key in the study of Alzheimer's disease. In addition, neuronal cultures are generally short-lasting (hours and days) and as such, it might not provide access to long- term compensatory events occuring in the living brain (weeks and months).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 the majority of the in-vivo two-photon imaging experiments, we normally label neurons in a sparse manner to ensure a high signal to noise ratio and thus typically study one neuron per mouse. To calculate the number of animals required, we used power calculations taking into account standard deviation from previous published studies and own pilot experiments, a significance criterium of 0.05 and a desired power of 0.9. Using these parameters, we have calculated a sample size of 15 mice (or neurons) per experimental conditions.

Although the estimated number of animals (4500) reflects the total number of animals in the protocols (1500 in Protocol#1 + 3000 in Protocol#2), in this project we will effectively use a total of 3000 mice. The animals in Protocol#2 includes the 1500 GM mice generated in Protocol#1 plus 1500 wild type mice.

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

Given that the main advantage of two-photon microscopy is the ability to chronically image the same neurons -or subcellular structure – over weeks and months, we can image and detect age-related alterations in the same animal rather than having to use different groups at different stages, ultimately reducing the overall number of animals utilised.

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

The same animals used for the chronic imaging experiments could be used for the behavioural experiments. In this way, we will not only reduce the number of animals required for these experiments, but this will also allow us to correlate changes at the cellular and behavioural level in an animal-to animal basis highly improving the statistical power of our 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.

In this project, we will use well-characterised model of Alzheimer's disease (AD), whose severity are considered mostly moderate as the animals develop the pathological hallmarks of Alzheimer's disease (e.g. amyloid plaque accumulation) and memory deficits; but without the most debilitating clinical signs of dementia (e.g. inability to eat or drink due to ataxia).

Regarding methods, the cranial window and in-vivo two-photon imaging approached used in this project was developed over 20 years ago and it has been optimised and refined since then.

Improvements in sterile surgical technique, diligent monitoring of animal physiology and support through the surgery, minimisation of trauma with precise tissue handling during surgery and post- operative care have all contributed to minimise pain, suffering, or lasting harm to the animals.

Regarding behavioural design, we have minimised animal's stress and suffering by setting a "gradual slope" for the behavioural training which includes handling to introduce the animal to the experimenter, habituation to the training apparatus and configuration. A proper training session with will not start until the animal is comfortable and confident.

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

As Alzheimer's disease progresses in an age-dependent manner - amyloid plaque deposition and loss in neuronal connectivity and memory deficits gradually accumulate over time - it is not possible to use animals at a more immature stage or others with short-lifespan (zebrafish or flies). Similarly, we will use longitudinal imaging to investigate sub-cellular changes occuring over time (e.g. weeks and months), which preclude the use of terminally anaesthetised animals.

We are using mice as an animal model as they preserve fundamental biological processes found in humans, they have a short gestation period, and several well-characterised transgenic models of Alzheimer’s disease are available. In this programme, we will use

well-characterised animal models of Alzheimer's disease that carry mutations found in human familial form of dementia and as a consequence, they recapitulate many of the alterations observed in humans in an age-dependent manner such as synaptic loss and cognitive deficits – phenotypes that are essential for meeting the objectives of our research programme.

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

Although the cranial window is an invasive procedure, it has been refined over the last 20 year to minimise distress and facilitate recovery. Improvements in sterile surgical technique, diligent monitoring of animal physiology and support through the surgery, minimisation of trauma with precise tissue handling during surgery and post-operative care have all contributed to minimise pain, suffering, or lasting harm to the animals.

In addition, we have introduced post-operative care to minimise welfare cost to the animals including:

1) placing the animals in cages separately which are on a warming plate (37C) during recovery and monitored continuously until they regain consciousness, and then hourly for the next few hours. 2) monitoring of changes in body weight, food and water consumption, and presence/absence of normal defecation and urination will also be undertaken; checking all wounds for signs of infection, inflammation, and other complications. Animals are euthanised if they display signs of stress e.g. hunched posture. 3) Daily health observations will be recorded from the duration of the recovery and will be collected in laboratory notebooks; If necessary, we will ask assistance of a veterinarian for resolving any animal care problems.

During behavioural training, we have taken the following considerations to minimise animals' stress and suffering in behavioural design: 1) The animal will be given 'rodent toys' in the home cage in addition to regular nesting material. These toys may include wooden tubes, running wheel etc. This allows the animal to be familiar with the type of body posture and activities in later training sessions as early as possible. 2) we have set a 'gradual slope' for the behavioural training including firstly a 'handling' step, which introduces the experimenter to the animal, building a bond of trust by daily contact. Second, the animal has a habituation period to adapt to training configuration, the apparatus, the time spent on the apparatus, and reward delivery. A proper training session will not start until the animal is comfortable and confident. 3) We design cognitive tasks that maximally uses the animal's innate behaviours, such as running and foraging. This will significantly reduce animal's 'learning' difficulty as well as time-spent, and therefore increase animal's wellbeing. 4) As water restriction is challenging for the animals, requiring rigorous monitoring of animals' health and hydration status, water control will be accomplished using free home-cage access to water made slightly sour by a small amount of citric acid (CA; 1-2%).

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

We are actively exploring current publications on best practice guidance on the Laboratory Animal Science Association or LASA website (https://www.lasa.co.uk/current_publications/) such as "Guiding principles for preparing for

and undertaking aseptic surgery" or "Avoiding Mortality in Animal Research and Testing" to ensure experiments are conducted in the most refined way.

In addition, we are continuously exploring refinement methods in published work or in specialised research animal training websites such as:

https://researchanimaltraining.com/ https://www.nc3rs.org.uk/
https://www.understandinganimalresearch.org.uk/
We also consult other useful reference books such as "The Design of Animal Experiments" by Michael F W Festing to design experiments more effectively and thus maximize the chance of getting scientifically valid results. For advice on administartion of substance we may turn to the report "Refining procedures for the administration of substances" by Morton et. al., 2001 and for the generation of GA animals to the report "Refinement and Reduction in production of genetically altered mice" by Robinson et. al., 2004, both publications by the BVAAWF FRAME RSPCA UFAW Joint Working Group.

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

We will stay informed about the advances in the 3Rs by attending informational events provided locally and provided by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs). In addition, we will stay informed by regularly visiting the NC3Rs webpage (https://www.nc3rs.org.uk/).

23. Understanding mechanisms of bacterial pathogenesis for drug development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Bacterial pathogens, Infection, Antimicrobial agents, Immune response

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?

Bacterial infections, particularly infections caused by antibiotic resistant bacteria, such as the hospital associated bacteria MRSA and Clostridium difficile are a major clinical challenge. There is a need for better and more effective drugs. The aims of this project are to identify pathways by which such bacterial pathogens cause infection and to discover new antimicrobial agents which can effectively block 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?

Antibiotic resistant bacteria cause serious infections which are extremely hard to treat with available drugs. In 2019, globally, there were ~5 million deaths associated with antibiotic resistant bacteria, with respiratory infections responsible for over 1.5 million deaths. Hence there is a need to develop more effective antimicrobials against such antibiotic resistant

bacterial pathogens. In order to develop effective antimicrobials one needs to understand how these pathogenic bacteria establish an infection. We aim to identify and characterise the mode of action of the key bacterial proteins that control infection, and also identify host factors and immune pathways that protect against such infections.

Along with providing insight into the biology of host-bacterial interactions, data obtained from this work will be valuable for the effective design of new drugs against hard-to-treat bacterial infections.

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

The end of this project we expect to have

identified of novel drug targets through the identification of bacterial and host pathways that are responsible for infection

contributed to antimicrobial development through testing newly discovered agents

published data generated from this programme in peer-reviewed journals (that support the ARRIVE guidelines) with the widest readership and with the highest possible impact, and communicated to the academic community through presentations at international conferences.

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

Academic benefits: In the short term, this research, which will use novel *in vitro* human and *in vivo* murine models of infection will advance our fundamental knowledge of infection biology. The findings of our research will be of significance to microbiologists interested in clinical and basic aspects.

Engagement with Clinical Research groups Collaborations will be initiated with clinical researchers with an interest in hospital-acquired infections during the course of this programme. Results from this research will be discussed at group meetings involving clinicians. The planned research will be expanded where possible to include emerging antibiotic resistant clinical isolates.

Benefits to the Commercial sector: In the medium and long term, the data acquired from our studies could lead to the discovery of new drug and vaccine targets as well novel antimicrobials against antibiotic resistant bacterial infections.

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

We will collaborate with groups working on similar infections to share knowledge, good practices and seek technical advice when establishing new approaches.

We will ensure that new results from this study are communicated to the academic community at local meetings and international conferences.
Data generated during the study, including negative data will be published in peer-reviewed journals with the wide readership.

Species and numbers of animals expected to be used

- Mice: 2250

Predicted harms

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

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

A whole animal model is essential for this programme as an intact immune system is required for investigating bacterial pathogenesis. Mice have been widely used to model a number of respiratory and gut bacterial infections and are preferable due to ease of manipulation and availability of molecular reagents. A murine model of infection will be used to investigate bacterial mutants and study effects of inhibitory agents.

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

Typically, mice would be inoculated with appropriate doses of bacteria (respiratory or gastrointestinal pathogens) either by an oral route or by intradermal (ID), subcutaneous (SC), intraperitoneal (IP), intranasal (IN) or intravenous (IV) injections. The route of inoculation will be decided based on the type of pathogen/ infection being studied and the key biological questions being asked, for e.g. For a gastrointestinal pathogen, an oral route may be chosen. Mice may be infected for different times (ranging from 1 day up to 6 weeks, depending on the type of infection), and regularly monitored for visible signs of illness. We will ensure that the illness they experience does not exceed moderate severity. For some experiments, prior to infection, mice may be injected using an appropriate route like SC or IP with antibiotics, labelling agents (for imaging), or post-infection, they may be injected using an appropriate route like IV or IN with novel antimicrobial agents. Where necessary, we may withdraw blood from a superficial vein (maximum twice a day, not more than 7.5% blood volume) to monitor markers of infection.

While typically a mouse will be subject to 1-2 administrations/day, the total number of procedures to a mouse will be limited to 4 procedures per day. A typical experiment that spans over 2 weeks will involve a mouse being subject to 1-2 injections per day with either 4 bloods samples/ week or 4 anaesthesia- requiring imaging sessions/ week.

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

The effects of infection on mice will depend on the type of pathogen used to infect mice.

Mice will be monitored for any external symptoms of illness including ruffled fur, slow movement, skin lesion formation or diarrhoea. Weight change is not a guide to the outcome of infection but may be used as a general objective indicator of disease: individual animals will be weighed at the start of the infection and every day after and weights will be recorded.

Whilst every effort will be made to reduce suffering there may be some unexpected instances where an animal is found to have more serious adverse effects (i.e. reduced

mobility). Any animals that are suffering or showing adverse effects that cannot be ameliorated will be euthanized immediately using a schedule 1 method to minimise 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)?

We expect that the procedures conducted will result in effects no more than moderate severity.

It is not expected or intended that animals develop adverse signs including piloerection, abnormal respiration, reduced activity or mobility, 15% weight loss. Any mice showing a weight loss of 15% of the original weight will be immediately killed by a schedule 1 method.

We expect that overall 55% mice will suffer moderate severity, 27% animals would have mild severity and 18% will be sub-threshold.

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

- Killed

Replacement

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

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

Assessment of the establishment and progression of infection is only possible in whole animals that can display the full extent of the pathogenic process. Thus, a whole animal infection model is necessary to identify bacterial factors important for infection as well as assess the host responses towards bacterial infection. Additionally, effectiveness of antimicrobial agents need to be assessed in a whole animal infection model to demonstrate potential for further drug development.

We will however continue to review relevant literature and attend scientific meetings of relevance to determine whether there are alternatives to animal experiments in this context.

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

We have used a number of *in vitro* surrogates to study infection, such as growth assays, cellular infection assays using human cell lines, and 3D human cell infection models. We also use *in vitro* cell cytotoxicity assays to ensure that antimicrobial agents have minimal toxicity. We use these in the laboratory to reduce the number of strains or antimicrobial agents that we test in animals.

Why were they not suitable?

We have been unable to completely replace an animal model with *in vitro* tissue mimics or cell lines mainly because these models lack a functional immune system, which is necessary to understand the processes involved in bacterial pathogenesis.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 the studies we have done thus far which is typically 10-15 mice/ experiment. We have used statistical programmes such as G*power to determine the minimum size that is required to see a statistically significant difference between groups. We have calculated the numbers based on the numbers of strains we would like to test, control groups to be included and the minimum number of animals needed to achieve statistically sound data.

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

We will first screen the bacterial mutants or isolates in a range of *in vitro* assays that we have available in the laboratory. Only the mutants that have interesting phenotypes will be carried forward to a murine infection model.

Similarly, when testing antimicrobial agents, only agents that are inhibitory in laboratory infection models and that are non-cytotoxic as determined by cytotoxicity assays *in vitro* will be tested for antimicrobial activity in animal infection models.

NC3R's research design tool will be used to optimise the experimental design of each experiment as it is being undertaken to ensure that the ARRIVE guidelines are being met in terms of reporting of experiments.

The data will then be used to perform power calculations to estimate the correct numbers of animals required for generating statistically valid data in collaboration with a qualified statistician. The minimum number of animals necessary to provide statistically robust data will be used in each experiment. This will eliminate the need for repetition of experiments, each of which would require the use of control animals.
We will use a single strain of mice wherever possible to reduce experimental variation.

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 for both infection and toxicity experiments will first be conducted with small groups of animals based on published literature. We will share tissue across different projects where possible.

Refinement

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

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

Mice are considered the standard non-human primate models for studying bacterial infections, with mice being the most accessible small animal system. There is a considerable body of literature with respiratory and gut pathogens using mouse infection models and this will provide substantial comparative data to compare the results of this project with.

Pilot studies will be first performed to determine the optimal bacterial dose or dose of compound tested. All experimental animals including uninfected controls will be monitored at frequent intervals during the pilot experiments and the frequency of daily health checks will be increased as the symptoms of infection become more pronounced. A daily record will be made of the disease state measured by the externally observed symptoms of disease of each individual animal; this detailed scoring system will be used to prevent animals reaching excessive suffering. Over recent years using this scale system, describing the symptoms of disease has been refined to more accurately predict animals likely to succumb to infection. This has reduced the number of animals exhibiting severe symptoms following infection.

While the weight change in the animals is not an indication of the likely outcome of infection this will be included as a general objective guide of the health of animals. Individual animals will be weighed at the start of the infection and daily after and weights will be recorded. Mice can recover from significant weight loss of 15% of their initial starting weight, however to reduce the risk of any additional distress to the animals, any mice showing a weight loss of 15% of the original weight will be immediately killed by a schedule 1 method.

Whilst every effort will be made to reduce suffering, there may be some unexpected instances where an animal is found to have more serious adverse effects (e.g. reduced mobility). Any animals that are showing adverse effects that cannot be ameloriated will be euthanized immediately using a schedule 1 method. The numbers of these animals will be monitored continuously to reduce suffering as far as possible. The pilot experiments will also help us define the best bacterial/ compound doses and will avoid unnecessary suffering. We do not expect more than 5% animals to show signs of suffering or distress requiring immediate euthanasia.

We have recently introduced a liquid/semi solid food for the mice after they are infected as this helps their well-being, reduces weight loss during the infection.

Where possible we will use non-invasive live imaging to monitor infection under anaesthesia. As a further means of refinement, physical restraining methods, which will avoid anaesthetising animals altogether, will be considered. When imaging with restraint animals will be habitualised to this before start of the experiments.

Monitoring freely behaving animals in specialised cages (for e.g. bioluminescence) will also be considered.

After every piece of work we will continue to critically appraise what we do to seek out ways to improve our models to reduce harm to animals.

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

The project aims to study the mammalian host responses to a whole bacterium, so that we are able to identify key proteins and pathways both in the bacterium and the host that can be drug or vaccine targets. Thus, the use of an adult mammal with a functional immune system is necessary for studying how bacteria induce host responses during infection. Hence, we are unable to use less sentient animals for our studies.

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

Monitoring: We have a very strict monitoring schedule for any procedure we do, especially for pilot studies while establishing new infection models. All experimental animals including uninfected controls will be monitored at frequent intervals during experiments and the frequency of daily health checks will be increased as the symptoms of infection become more pronounced. A daily record will be made of the disease state measured by the externally observed symptoms of disease of each individual animal.

Low stress-handling methods: To reduce stress levels in the mice on the day of the procedure, mice will be handled by the person prior to doing the procedure.

Reward-based positive enforcement: After we initiate an infection, we provide liquid or gel food in the cage which may help the infected mice lose less weight.

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.

24. The biology and ecology of mammals

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
- Protection of the natural environment in the interests of the health or welfare of man or animals
- Research aimed at preserving the species of animal subjected to regulated procedures as part of the programme of work

Key words

Marine mammals, diving, behaviour, health, population dynamics

Animal types	Life stages
Harbour seal	juvenile, adult, pregnant, aged, 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 purpose of this project is to understand the key factors that influence how phocid seals succeed or fail in UK waters by:

Increasing our knowledge of their behavioural and physiological adaptations to an aquatic existence.

Investigating the reasons for declines or increases in abundance.

Determining the variation in individual characteristics that influence their distribution, reproductive success, and survival.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 overall aim of the programme is to investigate how environmental and anthropogenic change impacts on the ecology, physiology and population dynamics of UK seals. Information on seal abundance, distribution, behaviour and health will be used to provide advice to government departments and conservation agencies on the management of UK seals.

There is a fundamental need to understand how marine top predators such as seals are likely to respond to rapid ocean changes and the sort of ecosystem shifts that are now being observed. The dynamics and abundance of the two UK seal species have changed dramatically in the last 10 years. Grey seal populations have increased or begun to stabilise whereas common (harbour) seals have experienced dramatic declines of up to 90% in some regions. The reasons for these changes remain unknown.

The work will thus focus on determining the mechanisms and processes that lead to the observed dynamics of the seal populations around the UK. It is based on having a fundamental understanding of the biology and ecology of UK seals and requires basic scientific underpinning studies which can provide data on how seals respond to change.

Work will comprise studies at different scales, including individual animals, colonies, and populations. By combining information collected from different approaches at the individual level, population models will allow us to predict responses to environmental change and increased human activity in the oceans (for example, investigating the effect offshore renewable energy developments, increases in ocean noise, changes in contaminant and toxin inputs, changes in weather and climate patterns and interactions with fisheries).

One hypothesis for the decline in UK harbour seals is an increase in competition for limited resources with grey seals. Knowledge of how these two species use the ocean and land is crucial to addressing this question. Sophisticated tracking devices will allow us to investigate the fine scale use of the marine ecosystem by both species at the same time.

Detailed studies of how the two species use their breeding and haulout sites, the factors affecting their choice of breeding colony and behaviouoral interactions between the species will continue to be of critical importance to predict the impact of future environmental change.

Defining seal habitat requirements is a key component necessary for the conservation and management of the species. Defining the requirements that allow animals to successfully feed, breed and rest will be used by policy makers to fulfil this conservation objective.

We also require detailed knowledge of the foraging behaviour of the seals including measurements of the effects of dive duration, activity levels, season and development on energy requirements and foraging capabilities, and on how diet can vary.

The work focuses on the two species of seal that are found in UK waters (grey and harbour seals). These are both listed in Annex II of the Habitats Directive. In addition, they are covered by the Conservation of Seals Act (1970) and the Marine Scotland (2010) Act. The results from these studies will be used to inform policy makers about the management and conservation of these seal species.

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

Outputs will include new data, a series of peer reviewed publications, and statotory advice to Government and stakeholders.

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

This project is a response to the needs of government and statutory conservation agencies to maintain a high level of up-to-date information about seals around the coasts of the UK. This need is driven by three main processes: (i) seals, like other marine mammals, are increasingly seen as important indicators/symbols of the state of our seas. They are at the top of marine food chains and therefore experience outcomes and pollutant flows of many anthropogenic effects; (ii) seals are viewed by some stakeholders as a pest species within the UK but, on a larger European scale, they are seen as rare species that require protection that will conserve their critical habitat. This leads to conflicting interests in the debate about how best to manage seal populations and this debate needs to be informed by high quality information about the interactions between seals and the environment, and the vulnerability of seals to changes in that environment within the UK; (iii) seals are likely important parts of the structure of the marine ecosystems surrounding the UK. They are clearly vulnerable to natural environmental and prey fluctuations and one species is declining rapidly in some parts of its range for as yet unknown reasons. As top predators, they are also potentially important drivers of ecosystem dynamics. The work in this study will allow us to assess their interactions with ecosystems, determine the effects of human disturbance, and help to inform the development of management decisions aimed at improving animal welfare and conservation.

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

Scientific publications: A portfolio of high-profile scientific publications will be produced throughout the project. Each scientific publication will also have a non-technical summary drafted, to highlight the relevance of the findings to a non-scientific audience. Internationally, we will disseminate findings to a global audience of scientists, policymakers, and industry through presentations at international workshops and conferences as well as to the general public via social media summaries of these papers.

Engagement with policy makers, regulators, and industry. The project license holder will engage with Government and key stakeholders involved in the understanding and managing seal populations and will attend appropriate meetings to ensure that 1. Findings and advice that arise from the data are communicated effectively to policy makers and managers; 2. The outputs are fed into advice and receive feedback in a timely fashion; 3. Opportunities for uptake and dissemination of the findings, cross-disciplinary collaborations and career development are maximised; and 4. New needs identified through dialogue can be incorporated into the work quickly, where possible. This will be achieved through the preparation of briefing papers for stakeholders and regulators.

Public engagement. The project license holder will work with our Institute's Press Office to engage with the public by contacting science and environment correspondents from national newspapers. We already have a strong track record in contributing material leading to articles on the behaviour and physiology of seals. We will use existing relationships to engage with relevant TV and radio shows and podcasts.

Species and numbers of animals expected to be used

2175

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 objective of this research is to understand the biology and ecology of harbour and grey seals. This can only be achieved by studying all age classes of both species.

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

Seals will typically be captured using nets or traps on or close to haul outs, breeding grounds or feeding areas. They will be lightly sedated during initial handling and will then be weighed and anaesthetized. Each seal will then have a series of morphometrics measured (e.g., length and girth) and, depending upon the specific study, may have blood or tissue (e.g., blubber, skin, tooth) samples taken, a telemetry tag applied to the fur, and an appropriate mark (e.g., tattoo) applied. They will then have a short recovery time (typically up to 30 minutes) and, following a successful ‘fit for release’ assessment, they will then be released back into the wild.

For studies at the captive facility, seals will typically be captured using nets or traps on or close to haul outs, breeding grounds or feeding areas. They will be lightly sedated during initial handling and transported to the captive facility. Seals will then undergo a period of acclimation to the pool facility which will typically last for 2-3 weeks. Each seal will then have an appropriate mark (e.g., tattoo) applied, and will take part in a series of studies over a period of several months. These will typically include (i) anaesthesia, (ii) blood, faecal, and tissue (e.g., blubber) samples being taken, (iii) the application of telemetry devices or data loggers (external by gluing to fur, and (iv) carrying out foraging experiments in a 42 by 6 by 2.5m experimental pool for periods of several hours, during which the surface of the pool will be covered by panels to restrict access to the surface to a breathing hole which functions as a respirometry chamber. The seals will be free to swim to and from an underwater feeding station. Following a successful ‘fit for release’ assessment, they will then be released back into the wild.

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

All animals are only likely to experience short-term mild pain, suffering or distress during the project, and it is highly unlikely that these will lead to a significant impairment in their

well-being or general condition. Further, any pain or suffering experienced by an animal will be, at worst, only slight or transitory and minor such that it will likely return to its normal state within a short period of 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)?

Mild. 100%

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

- Set free

Replacement

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

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

We need to study the behaviour and physiology of the animals directly because there are no alternative approaches that will allow us to answer questions about natural and human impacts on seal populations around our coasts and how they are likely to be affected by increasing pressure on the marine ecosystem. These studies will be carried out in conjunction with sophisticated statistical and mathematical modelling of seal population dynamics and movements so we can predict how their distribution and abundance may change in future.

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

We will continue to develop appropriate in vitro approaches so that, for example, the effects of particular contaminants and toxins on seal cells may be assessed.

Why were they not suitable?

The objective of this research is to understand the biology and ecology of seals. This can only be achieved by studying them in the wild or temporary captivity. Our integrated research programme will culminate in mathematical models. To be accurately predictive such models must be based on empirical data. However, where appropriate in-vitro models continue to be developed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

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

Pups are tagged to investigate survival and recruitment into breeding colonies. Estimating this requires large sample sizes due to high initial natural mortality rates in seals during their first year of life. Subsequent recruitment is often delayed until age 10, so further natural mortality will reduce the number of marked animals returning to the breeding site for study. We anticipate tagging up to 400 new pups across the various breeding colonies each year to carry out this aspect of the project, typically <10% of the pup production for the colonies of interest.

The captive facility may take in and release up to 20 animals per year depending on the types of studies being carried.

All other objectives will be satisfied by studying up to 250 additional new animals each year.

The total numbers of animals over a 5-year project period will thus be no more than 2175 for each species.

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

At each stage the study designs are discussed with our Establishment statisticians to ensure sufficient statistical power and biological meaningful results are achieved using the minimum number of animals. The number of animals artificially marked has been reduced by increasingly accurate photo- identification methods.

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 combine animal captures and procedures for the range of studies in the project, thus reducing the numbers of animals that would need to be captured if the studies were carried out isolation.

Refinement

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

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

Grey and harbour seals are chosen due to legislative drivers.

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

The objective of this research is to understand the biology and ecology of seals. This can only be achieved by studying them in the wild or in a temporary captive environment. Our integrated research programme will culminate in mathematical predictive models. To be robust such models must be based on empirical data and validated. However, where appropriate in-vitro models continue to be developed.

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

All procedures constitute international best practice, improved with veterinary advice. This process continues by review and refinement among licence holders, ensuring standards continue to improve. Additional local anaesthesia is now being used in addition to sedation during sampling procedures and handling times kept to a minimum.

Operant conditioning will be used as a means of training behaviours through positive reward. Behaviours trained using this method will reduce the requirement for handling, are key to achieving some research goals and provides important enrichment for the animals.

Work is underway to refine marking techniques; these depend upon the longevity of the required mark and the required visibility of the mark, the most refined being chosen as per scientific goals. Tattooing is the most refined marking technique used to date but is not visible from range so a flipper tag may be applied if re-sighting is included in the study.

In addition to testing sensors and devices, we are developing a bespoke, remote animal-borne blood sampler with data-logging functions for phocid seals. This will enable us to collect blood samples that are not affected by handling stress, which is particularly important for measuring baselines or where samples are required immediately after experimental exposure without the need for direct manipulation.
We have developed an anatomically realistic physical seal model that allows users to train animal handling and key licensed procedures. Tuition with the anatomically accurate model, combined with the skills and resources developed over many years enables competent staff to demonstrate best practice in a controlled, low-pressure environment where time can be taken to practice without any impact on live animals. End users refine techniques through repetitive use and tuition from experienced staff.

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

As far as possible, we will follow published guidelines during the planning and implementation phases of the project, including the ARRIVE (https://arriveguidelines.org/arrive-guidelines) and the PREPARE (http://journals.sagepub.com/doi/full/10.1177/0023677217724823) guidelines.

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, we will meet the NC3Rs Regional Programme Manager, and attend Regional 3Rs symposia.

25. Stem cells and liver regeneration

Project duration

5 years 0 months

Project purpose

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

Key words

Liver injury, Regeneration, Therapy, Stem cells

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo
Spiny mice (Acomys cahirinus)	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 improve the regenerative capacities of the liver and use this knowledge to create new technology for therapeutic purposes.

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

Liver disease is on the rise.

In the United Kingdom, deaths due to liver disease have increased by 400% since 1970. Every day, over 40 people die from liver disease in the UK. Liver disease is the third leading cause of premature death in the UK and three quarters of people are currently diagnosed at a late stage, when it is too late for lifestyle changes or intervention (British Liver Trust, https://britishlivertrust.org.uk/about-us/media- centre/statistics/).

In Scotland, deaths from chronic liver disease account for 1 in 50 of all deaths, and treatment for alcohol-related conditions costs over £1M per day (Office for National Statistics, consulted on March 2022).

The only curative option for end-stage liver disease is orthotopic liver transplantation, but donor organ availability cannot meet demand and many patients die waiting for a suitable organ. Moreover, many patients with end-stage liver disease are not eligible for transplantation. Those who do receive a transplant require lifelong immunosuppression with the increased health risks this involves.

Therefore, developing alternative strategies to stimulate liver regeneration is a crucial goal for liver disease.

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

Our investigations under this license will allow us to identify:

Cell populations and molecular targets that influence liver regeneration under conditions of acute or chronic liver injury.

Biological pathways and mechanisms by which cell populations (such as hepatic progenitor cells or macrophages) interact with each other and with the surrounding liver environment to influence the development/resolution of liver injury and organ fibrosis.

Development of potential diagnostic technologies for organ injury and regeneration. We predict the ability to be able to detect changes in liver volume and function and alterations to circulating blood components or bile following therapeutic interventions. These developments, whilst increasing our knowledge of the effects of our therapeutic interventions will also allow a reduction in animal use.

All our results will be published in peer-reviewed journals, enabling the scientific community to learn and advance.

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

Immediate benefits:

Scientific and technological advances that will increase the overall knowledge and capabilities of the field and the scientific community.

Medium benefits:

By identifying novel pathways, cell populations and signalling mechanisms that control liver regeneration we expect to influence these processes. Injury pathways and signalling mechanisms identified may also have potential applications in regeneration and repair outwith the liver, for example in fibrosis of other organs. Overall, our results will allow the rational application of both pharmaceutical and cell therapy strategies to influence overall organ regeneration and optimise the healing response following liver injury.

These pre-clinical strategies have the potential of being rapidly translated into the clinic.

Long term benefits:

Ultimately, we aim to improve and provide novel therapeutic options for liver disease patients. Patients with liver injury have very limited treatment options and it is therefore imperative to translate pre-clinical findings to a clinical setting. We are well positioned to capitalise on the clinical opportunities offered, as we frequently care for patients with advanced liver disease and we have been involved in clinical trials for novel liver disease therapies.

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

All data produced in the procedures covered under this license will be available via open access, peer- reviewed journals. We expect to deliver a number of publications in high-profile journals, conferences and patents that will leverage our aspirations to bring novel technologies to the liver disease patient.

Species and numbers of animals expected to be used

- Mice: 18000
- Other rodents: No answer provided

Predicted Harms

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

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

We will use rodents for this research, which are versatile and allow us to robustly reproduce the clinical conditions of liver disease. Using mouse models, we can replicate multiple liver conditions via genetic manipulations, dietary damage and surgical interventions or a combination thereof.

We have ample experience in the development of mouse liver disease models and can now induce specific conditions such as paracetamol overdose, primary biliary cirrhosis, fatty liver disease and end- stage fibrosis.

The spiny mouse (Acomys cahirinus) has developed an autotomic behaviour to escape predation by shedding up to 70% of its dorsal skin. Interestingly, the spiny can regrow back cartilage, muscle, skin and appendages (such as nails and hair) with minimal signs of fibrosis. It is therefore an animal model that exhibits accelerated and extensive regeneration. We aim to investigate which cell populations and biological pathways are responsible for this response and apply it to improve liver regenerative mechanisms.

We will investigate both acute and chronic liver injury conditions, which can span from developmental stages until adulthood. We will therefore assess rodents at all developmental stages.

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

Animals used in this project will receive a variety of regimes depending on the type of liver condition we try to modulate. For example:

Acute liver injury can be modelled by injecting a paracetamol overdose, followed by behavioural assessment and culling.

Chronic liver injury can be modelled using surgery (ischemia-reperfusion-injury, bile duct ligation), diets (DDC, MCD) and/or injection of substances (CCl4).

Liver regenerative responses can be measured via surgical interventions (partial hepatectomy that reproduce clinical resections), injection of substances or diets that promote hepatic progenitor cell response.

Therapeutic approaches can be explored by cell therapy interventions (intrahepatic, intrasplenic transplantation) or injection of substances (senolytic administration via oral gavage or intraperitoneal injection).

A typical protocol for modelling and treating biliary disease would involve: intraperitoneal administration of tamoxifen to activate cellular senescence in transgenic mice for 1 week, followed by a dietary model that reproduces biliary disease (DDC diet) up to 21 days and intrasplenic transplantation of hepatic progenitor cells.

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

Weight loss with dietary models (typically lasting no longer than 21 days). This will be controlled by monitoring weight loss and body condition. Weight loss will be observed for the duration of the experiment (majority of the weight lost expected to be around 5-18%).

Administration of substances in the diet may cause unpalatability and associated dehydration. Diet can be administered as a mash to facilitate the access to the food and avoid dehydration. Fluid replacement therapy may be used when the animal is dehydrated (as seen by lack of urination).

Pain following surgical procedures (up to 3 days post operation) will be significantly reduced using analgesia (applied during surgery) and placing the animals in warm cages with soft bedding.

Administration of substances such as paracetamol (APAP) overdose could cause temporary discomfort. Animal could exhibit clinical signs such as hunched posture, moderate piloerection, reduced spontaneous movement and reduced response to external stimuli from 4-8 hours post injection up to 24 hours after injection. A scoring system has been set up to monitor animal during those times. Animals are placed in warm cages with soft bedding and access to mash for support.

Post-surgical infections may occur after laparotomies but should be less than 1%. The risk of postoperative infection and delayed healing will be minimised by using aseptic techniques during the operation and regular postoperative checks to exclude potential sources of infection such as skin lesions and inflammation.

Anaesthetic level will be monitored during surgery (with deaths resulting from anaesthesia or surgical complications being uncommon, <1%). This will be minimised by correct control of inhalable substances and good maintenance of body temperature during surgery. Hypothermia will be prevented during surgery by using supportive heating and monitorization.

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

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

Severity varies depending on the procedure (from mild to moderate). Any unexpected deaths occurring for any procedures will be monitored and reported.

Breeding and Maintenance of GA mice: 90% Sub-threshold and 10% Mild

Mice (Protocol 2-9):15% Mild, 80% Moderate, 5% Non-recovery

Spiny (Protocol 10-16): 20% Mild, 75% Moderate, 5% Non-recovery

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

- Killed
- Used in other projects

State what non-animal alternatives are available in this field, which alternatives you have 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 increasingly recognized that the factors controlling liver disease and regeneration are complex and mediated through multiple cellular components and via multiple signalling pathways. For example, the cells that mediate regeneration can be either hepatocytes, cholangiocytes or hepatic progenitor cells depending on a complex interplay between the type of liver injury, the severity, the duration and the pre-existing condition of the liver cells.

We are increasingly realising that this response is absolutely dependent upon the cellular niche to occur. Therefore, although we do perform many in vitro experiments that complement and validate our in vivo work, the complex in vivo interplay cannot currently be modelled in vitro.

This means we must use in vivo models to study these areas of liver regeneration.

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

We make use of alternatives to animal whenever we can, including in vitro cell and organoid systems. Moreover, we have access to human donor livers discarded from transplantation, and we can perfuse them ex vivo to complement our translational research.

Why were they not suitable?

We have a strong program of in vitro work exploring the behaviour of cells in monoculture and/or in co- culture. For example, to examine signalling pathways between the macrophages and hepatic progenitor cells. We also perform analysis of human livers to analyse cell populations, gene expression or protein levels.

This work complements our in vivo work by regularly cross-validating the signalling pathways and allowing us to reduce and refine our in vivo studies, but does not replace the knowledge we can obtain with our in vivo studies.

As mentioned before, the level of complexity of liver disease (with multiple cell types interacting together in complex and evolving 3D structures) cannot be satisfactorily modelled using in vitro models alone nor can provide the required pre-clinical safety and biodistribution data of our proposed therapeutic approaches.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe

practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

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

We estimate the given numbers of experimental animals based on usage statistics of previous publications as well as information provided from other license holders at our establishment and national/international collaborators. The numbers required for upkeep and desired out-breeding of lines are derived from estimates provided by the animal facility. To stay updated on optimized experimental design, we will regularly seek guidance from biostatistical experts.

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

In the experimental design stage of the project, we have made plans to perform pilot studies where possible before planning for large cohorts. All experiments will be conducted according to the ARRIVE guidelines.

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

We will implement efficient breeding procedures and will additionally perform well-designed pilot studies whenever possible to assess the potential for publishable results.

We will additionally perform computational modelling and analysis of datasets to maximize the information extracted from our datasets.

Refinement

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

Which animal models and methods 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 rodents as an animal model to assess liver regeneration and novel therapeutic strategies including cell therapy. We aim to understand how liver regeneration works in the context of acute and chronic liver disease, which will provide a foundation to provide novel treatments.

To do so, we will use wild-type and transgenic mice without harmful phenotypes as animal models, meaning that rodents in the unperturbed state will experience no harmful effects.

We have also identified species-specific regenerative responses which require different models (e.g., the spiny mouse exhibits an accelerated fibrotic-free regeneration).

All these models are essential to recapitulate the clinical conditions, understand the biology of liver regeneration and advance towards a cure for liver disease.

To decrease the suffering and distress for the models, we have extensively validated measures of outcome for all the experimental protocols (e.g., number of hepatic progenitor cells in each area of tissue and biochemical assays of liver function such as serum albumin, transaminases and bilirubin). We have also included digital image quantification and gene expression analysis using quantitative real-time PCR to give an indication of levels of protein and gene expression at any given timepoints. This allow us to maximise the information gained from any particular experimental protocol.

To minimise the suffering of the animals we ensure good practices during surgery (aseptic technique), analgesia, active monitoring using body weight and body condition, which has proven to be very effective in our past experiments to assess the condition of the animals.

Any tissues generated from previous experiments are archived and stored appropriately, ensuring that experiments are not repeated unnecessarily.

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

Terminally anaesthetised mice do not allow us to recapitulate the clinical scenarios for liver disease, where the patients are monitored over the course of time. Moreover, most of the interventions that we use to generate liver disease are time-dependent (for example, dietary-induced liver disease evolves over the course of 21 days).

Immature life stages that are less sentient do not have a fully developed liver that responds to injury and/or therapeutics in the expected manner. Species which are less transient, such as zebrafish, also present a different liver architecture and has its own specific hepatic structure, not recapitulating a similar human liver pathophysiology.

Other animals, such as rats, are not suitable for some models to study gain/loss of function, as no suitable transgenic lines are available. In addition, the larger number of cells required for transplantation might limit the procurement of sufficient cells for our studies.

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

Dietary models (DDC, MCD, etc):

Daily check of the animals and weight assessment (three times per week for the duration of the experiment) will be used to monitor potential weight loss. If the animals show signs of discomfort, we will use mash and heated cabinets and/or heat pads. These measures help to maintain the welfare of the animal via facilitating their access to food and hydration source and regulating their body temperature.

For all our dietary models, bodyweight will be measured throughout the study (minimum three times per week). During weighing, mice will be checked for any clinical signs and adverse body condition including activity, signs of stress or pain (hunching, piloerection, social exclusion) and cachexia. Should any clinical sign or persistent weight loss be observed, mice will be closely monitored. In addition, one or a combination of the following interventions will be implemented:

- Diet can be administered as a mash to facilitate the access to the food and avoid dehydration.

- If signs of dehydration are observed (loss of appetite, no urination) mice could be administered subcutaneous sterile warm (37ºC) saline (0.9% NaCl) or Lactated Ringers Solution (LRS) as fluid replacement therapy.

- Mice could be maintained in a heated cabinet with soft bedding.

- Mice can be removed from the treatment for up to 72h to see if condition improves.

Should the condition not improve within 24h or weight loss reach 20% of starting body weight, mice will be culled by a Schedule 1 method.

Induction of liver fibrosis modelling chronic liver injury (CCl4 administration):

Approximately 5-10% of the animals experience moderate severity during the protocol (predominantly at late timepoints). Our regimes and monitorization allow us to produce reliable results within a moderate severity band.

Acute liver injury (paracetamol overdose):

The mice will be closely monitored using our current clinical scoring protocol, which we have agreed with the local NVS and the previous Home Office inspector. This clinical scoring system will use score sheets to assess mice at frequent intervals (from 4-8h post injection dependent upon strains, assess every 3h until 16h post injection). The scoring system is based on six phenotypic symptoms of acute liver injury: hunching, piloerection, neurological symptoms, responsiveness to touch, paleness and breathing rate.

Since the adverse effects and levels of liver injury in this model are strain-dependent (Harrill et al., 2009) we will perform dose titration studies when using new strains in order to ascertain the most refined dose compatible with the scientific objectives.

Surgically-induced liver injury:

Any studies involving surgical or invasive intervention will adopt appropriate pain management and post-operative care.

We will closely monitor anaesthesia and analgesia during surgery. Potential adverse effects will be minimised by correct dosing of injectable anaesthetics (or control of inhalable substances) by accurate weighing and maintenance of body temperature during and post-surgery.

Post-operative monitoring will take place daily with defined scoring thresholds (coat quality, skin tone, wound healing). Post-operative pain will be controlled by routine administration of analgesics as required. Excessive pain (recognised through modified behaviour, changes in facial expression, hunching, reduced movement or food intake) will be controlled with additional analgesics in consultation with our vets. Where this persists beyond 8 hours post-procedure, the animal will be culled by a Schedule 1 method.

Surgical sites will be monitored for signs of inflammation and infection. Wound dehiscence may be repaired on one occasion in consultation with the vets. Antibiotic prophylaxis may be administered post-operatively under veterinary advice.

Cell transplant and immunocompromised models:

We have refined our use of cell administration to immunodeficient mice using intra-splenic injection and we have improved the technique by performing a minimal incision on the flank rather than a complete laparotomy. With this new approach, mice recover faster and with minimal adverse effects.

Given that we sometimes transplant human cells to test their efficacy to treat liver disease, we use immunodeficient animal models that are permissive for the engraftment of foreign cells.Immunodeficient lines may have unpredictable death rates that can reach up to 6.7% in Rag2-/- IL2RG-/- strain and 8.3% in the AhCreMdm2 Rag2-/- IL2RG-/- line. Although these percentages are inherent to the intrinsic nature of the models, we will carefully observe these animals prior, during and after every experimental procedure, to ensure welfare of the rodent and minimise any associated risks.

Irradiation procedures:

As we fully reconstitute the bone marrow with stem cells post-irradiation, we observe extremely limited morbidity and mortality associated with this procedure. However, mice will be carefully monitored to ensure welfare is maintained.

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

We will stay up to date on best practice guidelines set forth and regularly updated from the NC3Rs website (Guidance on the Operations of ASPA - https://www.nc3rs.org.uk/).

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

We will stay informed about the advances in the 3Rs by regular literature search and the attendance to relevant conferences and seminars about new technologies and advances in techniques. We will also attend informational events provided locally and by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs). Team members with surgical oriented projects have the opportunity to attend an advanced

surgical course to keep up to date with the latest surgical advances and refine their skills to minimise harm.

26. The cytoprotective role of transcription factor Nrf2

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Bach1, Keap1, Nrf2, chronic disease, disease prevention

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?

We study a molecule called Nrf2, expressed in all cell types, which is transcription factor (a protein that takes the genetic information from DNA and changes it into the "messenger" molecule RNA so that a cell knows which genes to turn off and on). We want to find out how Nrf2 works to protect the cell from damage and understand how it reduces inflammatory processes. We will use this knowledge to help us and others to find ways to prevent and treat 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?

Turning on Nrf2 in cells (activation) can help to protect cells from harmful effects in many animal models of human disease, but the precise mechanism(s) are not fully understood. We need to better understand how Nrf2 works if we are to develop medicines that could target this protein in order to prevent and treat disease.

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

At the end of this project, there will be an increased understanding of the way in which the transcription factor Nrf2 works, including the mechanism(s) by which Nrf2 can be activated by small molecules, the protein target(s) of these small molecules, and how can Nrf2 be activated or inhibited. This will allow us to begin to develop new therapies for prevention and treatment of a range of chronic (long term) disease. We will share our findings by presenting the information we gain to other researchers at conferences and by publishing the information in peer-review journals, to allow the information to be publicly available.

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

Transcription factor Nrf2 and a second molecule called Keap1, which can "turn off" the Nrf2 protein (acting as the main "negative regulator") are now recognised as potential "targets" for new medicines, as they can alter how genes are turned on and off in cells. Several pharmaceutical companies are developing new cytoprotective agents (compounds that protect cells from damage) that work through targeting Keap1/Nrf2. Much of the current interest by the pharmaceutical industry in Nrf2 is centred on inflammation. Essentially all chronic human diseases contain an oxidative stress component. Oxidative stress is a condition that may occur when there are too many unstable molecules called free radicals in the body and not enough antioxidants to get rid of them. This can lead to cell and tissue damage and chronic inflammation, which also accompany ageing. As Nrf2 works to reduce oxidative stress and inflammation, we expect that activation of Nrf2 will reduce the clinical signs in long term diseases that have increased levels of inflammation (e.g. neurological conditions, including glioma, liver diseases, skin inflammatory conditions, osteoarthritis, and many others) as well as promoting healthy ageing.

In addition to helping people with chronic disease, Nrf2 activation could be also be helpful for people who, because of their genetic make-up, are likely to get diseases where oxidative stress and chronic inflammation have prominent roles in causing the symptoms of the disease. Indeed, in February 2023, the United States Food and Drug Administration approved the Nrf2 activator RTA-408 (Omaveloxolone) as the first and only drug for patients with the genetic disease Friedreich’s ataxia (a rare inherited disease that causes progressive damage to the person's nervous system and so causes movement problems).

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

To maximise the outputs of this work, we will work in close collaboration with colleagues in our own and other academic institutions, and with the pharmaceutical industry. We will participate in student exchange programmes (to share knowledge and experience with other institutions) and in addition to publishing our work, we will disseminate the new knowledge through participation in conferences and large international consortia, where we will work together with other researchers and organisations in order to help achieve our goal. We will adhere to the ARRIVE guidelines when reporting our results and publish even unexpected, 'negative' results, as we have been doing in previous projects.

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.

Nrf2 and how it is controlled by the regulatory mechanisms in cells is the same (conserved) between mice and humans. We use mice of all life stages and make every effort to use/develop mouse models that very closely represent what happens in humans and the specific human diseases that we are interested in. We are primarily interested in how changes in Nrf2 affect how easy it is to cause inflammation and need to be able to give potential drugs to see if such compounds affect the inflammatory responses of the animals. We also want to see whether the compounds work through some hitherto unknown mechanism(s) and this means we need to use a living organism as we don't know which interactions within the body might be important.

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

Most mice will be used in breeding programmes that let us maintain special "lines" of mice in which there are small genetic changes that mean that the level of Nrf2 activity is altered so we are able to use tissues and cells from these to look at how changed levels of Nrf2 affect the cells and the way they work. Some animals will be used in experiments to investigate how Nrf2 protects against inflammation and others will be used to work out the mechanism(s) of action of compounds that might potentially be useful as medicines by changing the amount of Nrf2 in cells (these are known as pharmacological modulators). These substances may be activators (turn up the levels of Nrf2) or inhibitors (reduce the levels of Nrf2).

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

We have generated specific mouse lines which either lack Nrf2 (or other substances that interact with it in cells) or possess it in super-abundance. We will test mice from these lines to see how easily the inflammatory pathways in the cells are to trigger when compared to "normal" mice that do not have any genetic changes. We will establish non-toxic doses of potentially protective compounds that activate or inhibit Nrf2. We try and dose animals in food or water whenever possible, but sometimes we need to inject them or dose them through a soft tube that goes down their throat into their stomach (gavage dosing). These procedures may make the mouse uncomfortable for a very short time but shouldn't interfere in any way with the animals normal behaviours and won't make them feel unwell. We may need to repeat the injections or gavage dosing several times.

Expected severity categories and the 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 nearly all our mice to experience no more than mild discomfort. Most of the mouse lines that we use look and act completely normally and will be used in breeding or will be humanely killed so that tissue can be taken to do experiments in the laboratory. A small number of our animals will receive injections or gavage, but in our experience, well-handled mice do not appear to be significantly affected by these procedures and return to their normal behaviours as soon as they are put back in their cage.

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?

Non-animal models on their own would not allow for the evaluation of the potential of Nrf2-modulating pharmacological agents to prevent and/or treat disease because they can't fully mimic how complex the body is.

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

Some parts of our programme will indeed be realised without using animals. In-silico methods, such as molecular docking and molecular dynamics studies, will be used for newly-developed compounds to understand how they bind to Keap1 or GSK3/beta-TrCP, the main negative regulators of Nrf2, for which crystal structures are available.

A large portion of the mechanistic studies related to this project will be performed using suitable cell culture and organoid systems (an organoid is a miniaturised and simplified version of an organ that mimics the key functional, structural and biological complexity of an organ). The methods used to work out how we measure Nrf2 activation or inhibition and its effects on pro-inflammatory responses will be first worked out in cultured cells (cells grown in the lab), such as macrophages from the bone marrow (macrophages are a type of white blood cell that surrounds and kills microorganisms, removes dead cells, and stimulates the action of other immune system cells).

Another set of cells we will look at in the lab to see how changed levels of Nrf2 affect them are microglia, which are cells in the brain that regulate brain development, maintenance of nerve networks, and injury repair.

Why were they not suitable?

The evaluation of the potential of Nrf2-modulating agents to prevent and/or treat disease can only be done in the context of the complexity of the whole organism, which is composed of many different cell types and organs that communicate with each other.

Reduction

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

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

This estimation is based on our previous experience with these models. We estimate using 1000 animals per year in maintaining the breeding colony, 400 of which will be used in

experimental procedures; a total of 1000 animals per year, or 5000 over the duration of the project.

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

We will design our experiments based on our previous research experience as well as after careful consideration of the various online tools available, including 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?

Wherever possible, as much use will be made of an experimental animal as feasible, within the confines of experimental design, in order to reduce the numbers of animals used. If different researchers are working on different aspects of the same system, then tissues/samples will be taken from the same animal in order to maximise the amount of information derived from a single animal, again reducing animal numbers.

To reduce the number of experimental animals, we will also harvest tissues and isolate primary cells, which we will grow ex vivo (in the lab) in culture. This will allow us to establish, from a single animal, a biological system that we can control, which we can expand and use in several different experiments, and be able to treat with various compounds (e.g. Nrf2 activators or inhibitors) or genetically manipulate (e.g. by stopping genes from being expressed by changing how the protein produced or altered) without subjecting the animals themselves to these treatments.

Refinement

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

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

Preliminary studies will use a short term well characterised model of inflammation induced by lipopolysaccharide to stimulate the inflammatory response and to investigate the effect of drug treatment on this response. The expected endpoints are well characterised in a wild type mouse that can be assessed using a scoring system if mice of a genetic alteration are found to show an increased reaction to the treament. In future studies, we will use a previously developed a mouse model of skin carcinogenesis, which recapitulates human cutaneous squamous cell carcinoma both genetically, epigenetically, and histopathologically.

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

To study the ability of potential Nrf2-modulating agents to affect inflammation and prevent/treat chronic disease, it is not possible to use terminally anaesthetised animals

because we need to look for medium and longer term effects and we can't use more immature life stages, because their Keap1/Nrf2 system is not yet fully formed/functional.

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

The animals will be monitored very closely for any signs of discomfort or pain. We will give compounds in food or treats if possible and where we need to inject or dose them by gavage we will use the minimum volume and fewest doses possible and animals will be acclimatised to handling before the study so that they don't find this upsetting.

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

The NC3Rs guidelines and power calculations from pilot studies will be used to refine all experiments. Published work on the models will be also carefully considered.

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

By regular reading websites (such as the NC3Rs) and published literature related to the 3Rs, and following advice from the Named Information Officer (NIO) and Named Veterinary Surgeon (NVS)/ NACWO (Named Animal Care and Welfare Officer).

27. Neuronal coding in the hippocampal system

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

neurons, oscillations, hippocampal formation, memory and its disorders, anxiety and anxiolytic drugs

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

We aim to make advances in understanding how the hippocampal formation, and the brain regions it is connected to, support critical functions such as memory and anxiety. We are also interested in making advances in understanding hippocampal-related models of diseases and 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?

Because we are very ignorant in our understanding of the underlying mechanisms and processes of HOW the hippocampal formation, and the brain regions it is connected to, supports functions such as spatial memory, memory for individuals of one’s own species, and anxiety.

This ignorance greatly hampers our ability to treat hippocampal-related diseases and disorders such as Alzheimer’s disease, Temporal Lobe Epilepsy, and Generalised Anxiety

Disorder. We need to be able to treat these diseases because they are a great burden to society. This ignorance is particularly acute as applies to Alzheimer's disease.

Alzheimer's disease (AD) is a crippling burden worldwide in terms of health services costs, and the loss to economies entailed in time-consuming, long-duration caregiving. Moreover, aside of economic costs, AD robs sufferers of their humanity, such as their ability to learn and remember things of great importance to them, and to their family/friends. Many of us know the pain of watching the brutal, inevitable decline of loved ones with AD, which go far beyond the decline of typical ageing. Over a century of AD research have brought us drugs whose beneficial effects are minimal. Why has intense AD research effort brought such minute benefits?

Different researchers give different answers to this question. One key explanation from the perspective of behavioural and cognitive neuroscience is that there is too large a gap between, on the one hand, molecular measures of intra/extra-cellular accumulation of e.g. beta-amyloid and tau, and of synaptic plasticity changes, and on the other hand, performance in 'memory tasks'. A potentially anti-AD drug given to rodents may reduce some pathology measures, & ostensibly seem to improve 'memory' in a spatial memory task, but that drug may in fact be reducing anxiety or inflexibility, or increasing motivation, a problem exacerbated by rodent-vs-human differences in pathological sequelae. How can we bridge this gap? We would argue that what is needed to bridge this gap are measures of functionally well-defined single-neurons, in early-AD-affected brain areas, bridging pathological measures and clearly-defined 'memory'. This is a crucial area of ignorance where we need more knowledge and understanding.

This kind of logic was behind the decision of the 2014 Medicine Nobel Prize panel to award that year's prize for the discovery of neurons with well-defined spatial functionality in the hippocampal formation (HF), the brain area known to degenerate early in AD, and the focus of the current Project. The cell types were place cells and grid cells. However, it has not been straightforward to find simple memory correlates in these cell types. We consider that we have found such a simple memory correlate in HF's output region, the subiculum, in neurons we called 'vector trace cells' (VTCs). These neurons appear to remember the distance and direction to objects and barriers in the local environment, even after they are removed. Thus these neurons can be used as a direct biomarker for the presence and strength of hippocampal memory. They can be used not just to test therapeutic approaches such as drugs, but also brain stimulation for example.

How might brain stimulation help? High profile studies in ageing humans have so far shown seemingly contradictory results: direct electrical stimulation enhances memory in one study, but worsens it in another. We argue that we must tailor brain stimulation more precisely, and to do so, we need to know more about, and appreciate the differences between, the processes involved in: setting up a memory in the first place ('encoding'); strengthening that memory ('consolidation'); and being able to call up that memory ('retrieval') when it is appropriate and helpful to do so. This requires investigation of memory mechanisms. Our work could suggest what brain rhythms, and at what phase of a brain rhythm, stimulation should occur at, for encoding, consolidation, and retrieval. Such work is only 15 years or so old, and there is still much to learn to ensure we can robustly improve patients' memory.

Finally, the working of these neurons, and a better idea of the set of cognitive functions that they serve, will help us to construct novel, simple, accurate tests to diagnose AD at earlier stages. We have national screening for diabetes, and for several different types of

cancer; why don't we have such screening for AD, given how devastating its effects are? Because we have do not have enough knowledge about the precise memory functions we should test for. That depends upon understanding the memory correlates of neurons in the regions attacked early on in AD.

And so it goes. Large governments could do big projects liking putting people on the moon, because the underlying physics was already known, and rest was engineering. Neuroscience is a very young science. We are not yet at the stage where we can reduce solving brain diseases to engineering. We are too ignorant for that.

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

As with previous Project Licences, the outputs are sets of new information, ie advances in knowledge, disseminated largely by publications in neuroscientific and interdisciplinary scientific journals, but also via talks at meetings and conferences.

Our objectives are to create new knowledge of mechanisms underlying memory and anxiety in health, the dysfunctions that arise in disease states, and ways these dysfunctions might be alleviated. Each output listed above delivers a step forward in at least one of those objectives.

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

Short-term benefits comprise the new sets of knowledge regarding hippocampal neuronal coding at the level of ensembles of single neurons, and oscillations. The coding we seek to reveal relates particularly to spatial and other types of memory, and anxiety.

Thus beneficiaries here include researchers pursuing fundamental knowledge regarding hippocampal coding mechanisms.

Medium and Longer-term benefits include: a) knowing what cellular and oscillatory properties to test in pre-clinical models of memory deficit (such as Alzheimer model rodents), and later in patients, so as to detect improvement by therapeutic agents (e.g. drugs) and manipulations (e.g. brain stimulation); b) improving assays for the anxiety-reducing efficacy of candidate anxiolytic drugs; c) knowledge that helps lead, ultimately, to tests which can accurately diagnose Alzheimer's disease at earlier stages.

Thus beneficiaries here include not only researchers pursuing fundamental knowledge, but also workers focused on pre-clinical models of disease/disorders and associated therapeutics, and potentially, clinical workers focused on humans. If that works well, then of course the ultimate beneficiaries are families with members suffering from hippocampus-affecting disease/disorders such as Alzheimer's disease and anxiety.

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

Naturally, we seek to maximise our outputs in many ways.

Dissemination of new knowledge: Papers, Conferences, Media Engagement Papers

We seek to publish in the best neuroscience journals possible, which have wide audiences, and wherever possible in an Open Access manner, sometimes at our own (considerable!) cost. Where this is not possible, we publish in a delayed Open Access manner.

Conferences

We present our work at National and International conferences. Media Engagement

We continue to engage with National and International press, and local and international radio/TV, and science journalists writing books.

Collaboration

We have frequently collaborated in the work enabled by our previous and current licences, to maximise the quality, scope, and impact of our studies. We have collaborations ongoing relating to past and current data. In conclusion, we are keen to foster collaborations, and am very aware that it helps to maximise outputs from our HO Licences.

Species and numbers of animals expected to be used

- Mice: 550
- Rats: 550

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 rats/mice because there is extensive knowledge of their neuroanatomy and physiology, and the techniques we intend to use have been designed for these animals and shown over 50 years to be successful. The discoveries of place cells, head direction cells, grid cells, and boundary vector cells were first made in rats, then later discovered in monkeys/humans (boundary vector cells are still to be shown in humans, but boundary-related coding in subiculum has been shown in two intracranial EEG studies, and one fMRI study). Clearly, rodent work translates to primates/humans, not only at a large region-behaviour correlation level (hippocampal lesions in all these species cause spatial memory deficits), but also at the level of types of individual neurons.

We focus on the adult stage.

For study of Alzheimer's Disease models, it is important to look at older ages, when the Alzheimer's Disease related pathology has accumulated over time so as to induce clear, measurable deficits in memory and cellular responses underlying memory impairment.

We use BOTH rats AND mice. Why both species?

RAT-SPECIFIC: We argue that rats are the best model for undertaking most kinds of electrophysiological work, especially in the spatial domain. Rats have been used extensively in these type of recording paradigms for over 40 years, and their brain anatomy and neurophysiology is generally well defined, better than most other similar mammals, and their spatial memory capabilities are well understood. The literature on rat spatial neurons is the richest literature of any mammal. They are eager to do the tasks, and good at spatial memory. Rats are more social than mice, and are thus very useful for

studying social-related memory. However, there are some situations where mice are more preferable models, as mentioned in the next paragraph.

MOUSE-SPECIFIC: We have argued above that rats are an excellent model for undertaking most kinds of E-Phys work. However, it is clear that when genetic manipulations enable more refined, precise, sophisticated approaches, as in optogenetics, mice offer a better choice. The variety of molecular techniques is so much larger in mice. Mice have been used a lot in recording paradigms for over 30 years, their brain anatomy & neurophysiology is well defined, better than most other similar mammals apart from rats, and their spatial memory capabilities are good & well understood. The literature on mice spatial neurons is the richest of any mammal apart from rats.

Mice tolerate head-fixed paradigms much better than rats. Two-dimensional virtual reality set ups for rats are very rare, and may not be so ecologically valid as those for mice. Accordingly, this licence restricts head-fixed paradigms to mice.

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

A rat or mouse will undergo surgery under sterile anaesthesia, typically lasting ~3-5 hours. During this surgery, one or two microdrives will be implanted on its skull, with electrode tips above target regions,

later permitting electrodes to be gradually lowered to the target regions of interest. Those regions are typically one or more regions of the hippocampal formation, such as CA1, Subiculum, and Entorhinal cortex. (These regions are affected in the early stages of Alzheimer’s disease).

After a period of recovery, typically 7-10 days, the animal will be taken from its holding room to a lab testing room, and screened for electrophysiological activity. In order to do this, we attach a headstage to each of the one or two microdrives, connected to wire which conveys the electrical signals to a digital oscilloscope and recording system. Typically, electrodes will be lowered over a period of days/weeks until target regions are reached, and then testing can begin.

Testing most typically involves electrophysiological recording during exposure to an open field such as a square/cylinder (typically 50cm high walls, ~1-1.5 m in sides/diameter for rats, smaller for mice).

During this exposure, the rodent forages for rice or milk. A trial for a rat typically lasts 20 minutes in a 1mx1m square box. A full testing session will involve several trials of this foraging task, lasting from 2-6 hours. The open field may contain objects and, occasionally, a concspecific. Rodents are typically very content to do this task.

Mice may also undergo a virtual reality task. This is somewhat akin to humans running on a belt in a gym while watching a TV-displayed world, such as a forest in New Zealand, whereby the faster the human runs, the faster the movement through the forest. This involves running on a polystyrene ball while computer monitors display a visual world. The mouse’s head is fixed, but in a manner that enables forward and sideways locomotion and turning. The VR world is typically one with walls and one or more objects, and we can change the geometry and features in it, to test spatial representation and memory. This is typically all done as a simple foraging task, or we can construct an appetitive task, whereby the mouse gets special reward for navigating to an unmarked spatial goal region.

Sometimes, the effects of an additional neural manipulation will be made, such as by injection of a drug (pharmacology, chemogenetics), or by switching on light (via optic cables as in optogenetics). These manipulations may be enabled by a further element of the original surgery mentioned above (e.g. injecting genetic material, inserting cannula or optic fibre implant), or they may NOT require that (e.g. injection of systemic anxiolytic drugs).

An animal is typically tested for 2-4 days per week when electrodes are in target regions. The average time an animal will undergo overall post-surgical testing is around 3-4 months.

Common variations on the above typical theme:

If we are testing 'anxiety' type responses, we may also expose the animals to classic tests of anxiety, such as the elevated plus maze, and novel open field. If we are testing 'social' type responses, we may also expose the animals to classic social interaction and memory tests, such as the three chamber test, which involves exposure to a novel and familiar conspecific.

One of our Protocols enables us to test 'deficit' models, typically transgenic mice, such as:

A) Alzheimer model mice, which will be expected to show, e.g., spatial memory-related, deficits at later ages;

B) mice thought to model aspects of autism spectrum disorder, which will be expected to show, e.g., social memory-related deficits.

Other than this aspect of the animals being (potential) models of diseases/disorders, testing is similar to that already described above.

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

SURGERY

All surgical procedures will be carried out under aseptic conditions and under balanced general anaesthesia. The animals will routinely receive analgesic drugs and antibiotic cover will be provided using suitable drugs or as advised by the NVS.

i) Rarely (est. < 0.5% of cases), animals following surgery may show distress as indexed by anorexia, hypoactivity, excessively hunched postures. If this situation persists for more than 5 days, the experiment will be terminated and the animal will be humanely killed.

ii) Unusually (est. < 2% of cases), following surgery the wound may become infected and cause discomfort. The NVS will be consulted and, if appropriate, topical or injectable antibiotics will be administered. If would does not heal appropriately, the experiment will be terminated and the animal will be humanely killed.

iii) Rarely (est. < 0.5% of cases), the microdrive implant may come loose and cause discomfort. If the implant becomes loose or broken, the animal will be briefly re-anaesthetised and the implant repaired or re-fastened once only. If this cannot be

successfully accomplished, the experiment will be terminated and the animal humanely killed.

iv) Rats and mice with skull-fixed implants typically recover very quickly after surgery, and beyond the first few days, show no behavioural changes or signs of stress. Occasionally (est. ≤ 10% of cases), the headcap (i.e. cemented implant and occasionally part of skull) will come off. In this case, the experiment will be terminated immediately and the animal humanely killed. We are gathering observations of potential markers (e.g. neck cringe) that accurately predict if the headcap will subsequently come off weeks later. If any marker has high accuracy (≥ 85% correct prognosis), the experiment will be terminated immediately and the animal humanely killed upon observation of the marker. This would minimise the adverse effect of the headcap coming off. In consultation with advice from the NVS and other Researchers, we are of course working to minimise the later development of this adverse effect at both the surgical implant stage (e.g. screw-related procedures) and afterwards (e.g. modifying cages).

BEHAVIOURAL tasks

i) An animal may sink in the water maze rather than swim. This is extremely unlikely (<1%) as rodents are good swimmers, but if it occurs the animal will be removed from that task.

ii) The behavioural testing procedures are not intended to cause pain. Only minimally aversive solutions will be given in the aversive conditioning tasks. In some experiments (< 5%) involving the interaction between two rats or two mice, the animals may fight or one may attack the other; if this occurs, a screen will be placed between them or the 2nd non-experimental animal will be removed. Animals showing signs of distress in any behavioural task will be removed from that task.

iii) In particular, although it has been demonstrated that rodents typically become well-accustomed to being head-fixed on a treadmill we envisage that the head securing procedure might initially cause distress in a subset of animals. Animals will be accustomed gradually to the head-securing procedure, over several sessions, and with the use of appetitive reward. Any animal showing distress (assessed by the occurrence of vocalisations and/or the animal struggling against the apparatus) will be removed from the head-securing apparatus immediately. **Additionally, any animal who does not become accustomed to the head-securing apparatus will be removed from this task.**

DRUG ADMINISTRATION

Where substances are administered (<15% of cases for protocols 1 & 2, and <40% of cases for protocol 3), a few animals (<10% of those administered) could show signs of distress associated with substance delivery such as scratching, piloerection, porphyrin staining, and cringeing. We will not attempt to ameliorate their initial signs, and they will be immediately removed from the experiment and humanely killed.

GENERAL POINT: POST-SURGICAL HOUSING (i.e. entire duration of procedure after surgery for surgery-operated animals)

As mentioned below in ‘Refinement’ there are potential adverse effects typically associated with classic post-surgical isolation housing. This is likely an issue with rats, who

are much more sociable than mice. With mice, there is actually good evidence that isolation housing reduces anxiety and stress (discussion of evidence & references below).

However, we have refined, and continue to refine, our procedures to minimise this, and we set out below in section 1 of 'Refinement' a complementary set of FIVE counter-acting measures ('mitigations'), notably including tickling and proxy social housing which we think greatly reduce the anxiety/stress as compared to that typically accompanying classic isolation housing. For mice, which are generally more aggressive and less social, we have three sets of mitigations.

Specific to Alzheimer model testing

In this protocol, we need to consider adverse effects more generally associated with ageing/AD models. We do not consider these will occur for the other models (e.g. part models of autistic characteristics), as we will not take them to the later ages needed for some Alzheimer-models. Note that our absolute maximum (estimated <8% of cases) protocol length is 18 months, for AD models only, is much earlier than some licences/models, which may extend beyond 3 years.

Background to Adverse effects

1) Importantly, it is increasingly clear that many of the sequelae associated with ageing in AD-models are greatly exacerbated by lack of enrichment and exercise, e.g. (Mehla, et al 2022. Dramatic impacts on brain pathology, anxiety, and cognitive function in the knock-in APPNL-GF mouse model of Alzheimer disease following long-term voluntary exercise. Alzheimer's Research & Therapy, 14) and similar articles. The stimulation provided by our behavioural tasks is rich and extends far beyond the stereotyped type of wheel running in this Mehla article and others like it, which was still highly ameliorating.

2) Importantly, as regards Alzheimer-models, unlike many others working on AD models, **we are NOT motivated to explore later stages of accelerated models of dementia.** Rather, we are interested in stages in rodents equivalent to the later EARLY and MIDDLE stages of AD, in what could be broadly considered, species differences aside, as later PRESYMPTOMATIC, and MILD COGNITIVE IMPAIRMENT, stages of AD, before the DEMENTIA stage. This is because our rationale focuses on the hippocampal formation, which is one of the first brain regions to degenerate in human AD. Our rationale is set out in some detail in BENEFITS and SCIENTIFIC BACKGROUND. Briefly, our goal is to detect early AD-related sequelae in hippocampal regions and behaviour, with a view to advancing preclinical study, including examination of potential therapeutics, and translation to tasks for diagnosing AD earlier in humans (which we have already begun, with some success, in collaboration with Clinical Psychologists and Neurologists).

Nevertheless, animals will be monitored for additional signs of illness and distress related to the ageing/AD model interaction, or long-term maintenance.

Common ailments in these adverse effects include but are not limited to:

a) Cataracts and ulcerations of the eye. Animals will be frequently checked for cataracts and other eye- related diseases. If an animal appears to be blind in both eyes, by identification of clouded eyes, the animal will be culled. If an animal is observed to have an ulcerated eye (by identification of reddened mucosa, weeping eye, cataract across eyeball) then it will be promptly killed.

b) Tooth issues, relating to poor body condition and suspected weight loss. Animals with tooth issues or poor coat condition will be weighed daily, and a record kept. Further, any animal that appears to be losing weight and/or body condition will be weighed daily, and a record kept. If the issue causing the weight loss is suspected to be inaccessibility to food, food pellets will be scattered on the floor. Wet mash and/or hydrogels will be additionally provided on the floor of the cage, along with extra wooden chews or toys to help prevent tooth overgrowth. Animals will be killed at the appearance of a loss of 15% body weight within 3 days, or weight loss without recovery within 24hrs, exceeding 15% of that expected for a healthy adult weight of that mouse strain, whichever is reached first.

c) Animals will be promptly killed if they show any of these signs listed below: Piloerect coat showing no improvement in up to 24 hours.

Skin lesions showing no improvement within 72 hours.

Skin tenting indicative of dehydration and loss of condition for more than 24 hours. Hunched appearance, not improving with 24 hours.

Unexpected tremors or head-bobbing. For lines with known mild tremors or head-bobbing, when these become debilitating (interfering with ability to feed and not improving within 12 hours).

Ataxia, interfering with the ability to eat or drink normally, or affecting balance causing stumbing and righting difficulties.

Abnormal response to handling - extreme lethargy or hyperactivity

Excessive aggression to cage-mates or animal technicians

Unexpected response to provocation

Unprovoked vocalisation

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

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

Rats

- Expected moderate (80%); Expected mild (20%). Mice
- Expected moderate (80%); Expected mild (20%).

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

- Killed

Replacement

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

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

Considerable work by others has gone into understanding the hippocampal network with and without synaptic plasticity using in vitro and computational models. It is nevertheless unavoidable that understanding the role of the hippocampus can only be achieved by the study of conscious freely- moving animals. Implantation of chronic indwelling electrodes in humans is only permissible in a very small number of clinical situations and for brief periods of time in very restricted environments and thus is impractical for research purposes. There is no non-invasive method of monitoring the firing patterns of groups of individual neurons in humans. Human neuroimaging techniques (fMRI, PET, MEG) lack either fine temporal resolution (PET and fMRI) or fine spatial resolution (MEG), and anyway deal with comparatively large sections of cortex (millions of neurons). FMRI and PET rely on metabolic changes, not that reliable an insight into neural coding.

We have collaborated with colleagues who devise computational models of hippocampal function and have used these to design experiments and predict their outcomes, but the models are extraordinarily simple in comparison to the complexity of the brain, and cannot substitute for experiments themselves.

The most notable direction of influence is from animal data to the models, and not vice versa. An illustration of the comparative poverty of modelling is provided by the Nobel Prize-winning discovery of grid cells in the entorhinal cortex (Hafting et al, Nature, 2005). Of the 100 or so models of the hippocampal formation’s contribution to navigation, at that time, not one model predicted these cells.

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

1) Computational in-silico models

2) Brain slices (derived from dead animals)

Why were they not suitable?

Computational in-silico models are extraordinarily simple in comparison to the complexity of the brain, and cannot substitute for experiments themselves. They play an important role in our field, but primarily to make predictions for those working with live animals and humans to test. Furthermore, obviously, they lack a direct link to behavioural output that changes as a result of learning (e.g. 'I will now move towards the place where I remember contained food reward last time').

Brain slices can be derived from dead animals and humans, and can be used to examine certain aspects of brain function in the living organism, such as anatomical connectivity (e.g. 'X type neurons excite 3% of other X type neurons, but mainly project to Y type neurons'), and cellular excitability. I read and value such work, and it informs my perspectives, but it cannot speak directly to the key questions that I ask. Only thin slices of small portions of a brain can be kept in a state sufficiently close to the real brain, so they are disconnected from the rest of the brain. They lack not only that link, but also, like in silico models, a direct link to behavioural output (e.g. 'I will now move towards the place where I remember contained food reward last time').

Reduction

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

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

The numbers are estimated per protocol, per experiment, typically per year, then summed. Breakdown as follows.

Numbers per experiment reflect decades of experience with success rates of e.g. targeting of electrodes.

RATS

Protocol 1: 350

70 per year, consisting of 3 expts per year of n = 13/14 each, and 2 expts per year of n = 15 each.

Protocol 2: 50

Two expts of n = 25 each, over the whole licence period, averaging n = 10 per year.

Protocol 3: 150

30 per year, consisting of 1 expt per year of n = 30 each (two groups of n = 15).

MICE

Protocol 1: 200

40 per year, consisting of 2 expts per year of n = 20 each.

Protocol 2: 50

Two expts of n = 25 each, over the whole licence period, averaging n = 10 per year.

Protocol 3: 300

60 per year, consisting of 2 expts per year of n = 30 each (two groups of n = 15).

Comments

Greater use of rats than mice in protocol 1 reflects larger size of rat brain for easier targeting, & better memory.

Greater use of mice than rats in protocol 3 reflects the much greater availability of transgenic mice than rats.

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

Our approaches to minimise this discomfort & suffering/information ratio are two-pronged. We seek both to maximise the amount of meaningful data obtained from each individual animal, and to refine our techniques so as to reduce discomfort/suffering.

By increasing the data obtained from each animal, fewer animals are needed per experiment. To give a simple example, instead of having one microdrive on each animal, we routinely use two microdrives per animal, and thus one animal generates the same amount of data as two animals. We will also start to use silicon probes which permit recording from hundreds, not tens, of electrodes.

Our technique of simultaneous, stable, chronic multi-site recording/archiving of neuronal ensembles, EEG, and ethological/spatial behaviour greatly improves the above ratio:

1) If brain region A in rat X and region B in rat Y respond differently to the same stimuli, it's possible that this occurs because rat X responds differently to rat Y, necessitating high numbers of rats. Recording simultaneously from two or more regions controls for inter-animal behavioural variability, thus reducing the number of rodents required.

By the same token, we are using as much within-animal pharmacological comparison as possible. For instance, each rat receives an anxiolytic drug and drug vehicle on different days, and these manipulations are compared (controlling for order). This uses fewer animals than in many drug studies where one group of animals gets the drug, and another group gets the vehicle control.

2) Because of the stability of our implants and recording procedures, we can record over long periods (days and months), obtaining a considerable data from each animal. Note that the discomfort of the animals typically decreases with time elapsed since the surgical operation. Arguably, the benefit of the extra information we obtain comes at a very low welfare cost.

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

We train lab workers to a very high standard, thus minimising errors in surgery, handling, screening and recording.

The richness of the information we obtain from each single trial. We obtain EEG data (lower-frequency, large-scale oscillatory activity that may relate to how different brain regions talk to each other), neuronal firing at a millisecond-to millisecond scale and for up to, say, a hundred neurons, spatial data (e.g where is the animal? ethological data (e.g. is it freezing, rearing, urinating?). All this information is stored.

The richness of these stored datasets eliminates the need for pilot work, thus further minimising animal numbers. Even our 'failures' can be highly informative. Sometimes, unavoidably, even the best surgery misses its intended targets. However, we can obtain information from another neural region when it is accidentally recorded from that can, and does, inform future work. Indeed, this can contribute to a future published study.

Because of the richness of our neural-measure datasets, I will often produce more than one published article from the same dataset.

Statistics

Statistical considerations, including power analysis using power analysis software (we currently use Gpower 3.1) further contributes to optimisation of numbers. These power analyses mostly apply to studies which compare groups: these would typically involve a manipulation group (e.g. drug, lesion, optogenetics) vs control group, or transgenic group vs wild-type. We do also consult colleagues with particular expertise in statistics. Power analyses tied to reading of past literature produces optimal numbers.

In our view, as regards electrophysiological experiments where control comparisons are typically within-animal, not across-animal, comparisons, it actually is chiefly good and careful experimental

design optimised from decades of experience in the field, and continual refinements allied to technical skill, that optimises the number of animals.

Reproducibility

I care deeply about reproducibility. One of the most obvious routes to wasting animals is publishing unreproducible work.

Reproducibility requires researchers to employ a range of motivations and abilities to ensure the combination of making findings which are, ideally, field-extending important advances, and yet fully replicable.

These include:

the desire to make findings that are replicable, i.e. a kind of honesty, over e.g. the temptations of career advancement;

meticulous care with executing procedures;

ensuring conditions are as similar as possible across re-sampling and different animals, and allying this to comprehensive note-taking, drawings/photographs, evidence collection of electrode set ups, stimuli, histology;

exhaustive reading of literature to sense what might be confounding variables so ‘intuition’ has a rich empirical base, and thus to minimise them;

and finally, and by no means most importantly, competent statistical understanding.

I have employed these throughout my career, and while the past is not a perfect guide to the future, am proud to say my record is excellent on reproducibility.

Essentially, ignoring very recent work where findings are yet to be studied by other groups, ALL the major findings in my career have been replicated. Arguably, the best guide to future reproducibility is a record of it in the past, rather than a checklist of promises.

Refinement

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

Which animal models and methods 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/ mice because there is extensive knowledge of their neuroanatomy and physiology, and the techniques we intend to use have been designed for these animals and shown over 50 years to be successful. The discoveries of place cells, head direction cells, grid cells, and boundary vector cells were first made in rats, then later discovered in monkeys/humans (boundary vector cells are still to be shown in humans, but boundary-related coding in subiculum has been shown in two intracranial EEG studies, and one fMRI study). Clearly, rodent work translates to primates/humans, not only at a large region-behaviour correlation level (hippocampal lesions in all these species cause spatial memory deficits), but also at the level of types of individual neurons.

We see our goal as to create the maximum amount of insight from a given unit of suffering, thus to keep increasing an ‘insight-to-suffering’ ratio, so to speak. Indeed, we would consider that we have delivered an increase in that ratio over successive Home Office project licences.

Our main method involves electrophysiological recording from rats and mice, under environmental and other manipulations. Our methods have been refined over decades, and continue to improve, so as to minimise pain, suffering, distress and lasting harm. Many of the refinements we have adopted are similar to rodent researchers in different fields, while some of our refinements relate specifically to the field of electrophysiological recording of ensembles of individual neurons from freely behaving rodents. We discuss some of these in the sections below. Here, I would like to outline a key set of mitigations regarding potential housing-related adverse effects that are associated with our technique in particular.

Housing and minimising stress

Our field typically uses single housing after surgery. This is in order to prevent damage to the stability of implanted microdrives. For the much cruder technique of multi-unit or local field potential recording, some degree of instability is acceptable. However, to stably record and follow the same individual neurons over a whole recording session, and sometimes across days (thus tracking long-term memory), one needs the very highest levels of implant stability.

Single housing, so other cage members cannot interfere with the implant, helps to ensure good implant stability. However, in rats, single housing is likely detrimental to mood, and tends to increase anxiety and stress. Here we want to emphasise that the risks of single-housing related adverse effects, which are likely to be most problematic when rodents are unstimulated for long periods in their home cage, are greatly mitigated in our hands in five ways.

1) The first mitigation, for rats, is our routine 'loving', affectionate, handling including playful stroking and tickling of rats over their experimental lifetime. This is provided by a single experimenter to whom they habituate, and is thought by some works to act as a kind of proxy for rough-and-tumble play, producing 'reward' type high frequency vocalisations (above those frequencies audible to humans.) There is ample evidence from other labs that human handling of even a less affectionate kind than ours reduces anxiety (e.g. Nunez et al, 1995, Physiology & Behavior; Vallee et al, 1997, Journal of Neuroscience; Costa et al, 2012, Journal of the American Association for Laboratory Science).

Importantly, there is good evidence from other labs for the beneficial effects of tickling**, e.g. that tickled isolated rats have anxiety as low as, or even lower than, untickled group-housed rats** (Hori et al, 2014, Hormones and Behavior).

We have been tickling rats for 30 years, well before the tide of now substantial evidence that it improves well-being.

2) The second mitigation is the rich environmental exposure to varying stimuli, often for several hours in the day once recovery from surgery is complete. This is akin to enriched environments, which are well known to reduce anxiety and stress, and boost immune status in mice and rats.

3) Third, we place cages in the holding room very closely to each other and at the same height, affording visual and auditory interaction, and at least some olfactory sampling. This enables some within-cohort social interaction and social cues.

4) For rats, we use fine-mesh to partition large cages into two halves, to enable 'proxy' social housing whereby two animals share one cage in a 'buddy' system. To our knowledge, we were the first single- unit recording laboratory in the UK (and probably the world) to offer this mitigation.

5) Fifth, though only mitigation for a subset of animals (<40%), some experiments examining social memory explicitly involve social interaction with conspecifics for many trials a week.

Mitigations 1 and 4 are in rats only.

Re mitigation 1, mice clearly prefer to avoid direct contact with humans.

Re mitigation 4, C57BL/6J mice, our typical mouse strain, have low social motivation, much less than rats, as one recent study by others shows (Netser et al, Nature Communications, 2020, 11). A review by others on models in neuroscience research concludes "In summary, whereas the majority of rats readily engage in social behaviour and find it rewarding, mice spend significantly less time interacting with a conspecific and many even find it aversive." (Ellenbroek and Youn, Disease Models and Mechanisms, 2016). Many studies and lines of evidence led to this conclusion. I just summarise, briefly, ONE line of evidence from three indicative studies cited by that review: communal rearing of MICE led to an INCREASE in anxiety in adulthood, whereas in RATS communal rearing led to DECREASES in anxiety.

Barkus et al (2022): head fixation, and fluid/food control

We will not employ head fixation in rats, but only in mice.

My lab is already employing the best practice procedures recommended in the paper on ‘refinements’ in Barkus et al (2022, J Neuroscience Methods), regards to handling, surgery, post-operative care, food and drink administration, and habituation to head fixing apparatus.

Importantly, we have not used any form of liquid deprivation in nearly 30 years of work on rodents, and have removed this unused option (in our previous 3 licences) from the present licence. In particular, we already ensure that there is adequate food and liquid given to mice undergoing head fixation. Of all our tasks, the head-fixation task in virtual reality is the task in which mice have the readiest access to food and liquid. This occurs in two ways:

1) the consumption of liquid foods such as soya milk throughout the task. Once a mouse is trained, the mouse receives liquid food such as soya milk typically at 5-10 second intervals. When the mouse is at the beginning of training this interval may be up to 30-60 seconds, but this is of course still very ready access.

2) the return to the ‘home cage’ which is moved to the laboratory during this kind of work, and which the mouse is returned to between any long sessions at the VR head fixation apparatus. A long session might be two trials of 40 minutes each, with a 30 minute interval. The cage has ad libitum water, and typically some remaining food in the home cage.

Barkus et al (2022): Not group housing mice

To my understanding, the only significant point of departure in our lab’s already-existing practices from the advice in Barkus et al 2022 concerns group housing for mice. Barkus et al (2022) advise: “Group housing following implants is strongly recommended to avoid the negative welfare impacts of single housing.” As indicated amply in the section above on ‘Housing and minimising stress’ we do use a buddy-cageing system for rats, a proxy for social housing, as we accept the arguments for social housing in rats. However, we do not do this for mice. In my view the argument for social housing in mice is far from compelling. Barkus et al (2022) cite two papers as supporting the argument for group housing: Kappel et al, (2017, Animals) and Liu et al (2020, Euro J Neuroscience). However, Kappel et al themselves say: “This review discusses whether it is in the best welfare interests of male mice to be housed in groups, or alone. We conclude that it is not possible to give general recommendations for good practice for housing male laboratory mice, as responses to single- and group-housing can be highly context-dependent.” As for Liu et al (2020), arguably this paper, whose effect sizes are not strong, shows the effects of lack of stimulation, rather than specifically the effects of single housing.

The mice had nothing to do but exist in their cages for 8 weeks without any opportunity to explore new environments, do interesting tasks and so on. They are held in their cages for all that time! That stands in some contrast to our work, which affords great opportunity to explore different environments, and do interesting tasks.

Moreover, importantly, as mentioned above, C57BL/6J mice, our typical mouse strain, have low social motivation, much less than rats (Netser et al, 2020), and Ellenbroek and Youn (2016) reviewed evidence from different studies showing that, unlike for rats, communal rearing of MICE led to an INCREASE in anxiety in adulthood.

In summary, we will continuously keep up to date with the literature, but do not consider the argument that mice (especially males, which we typically use) should be group housed is well-evidenced. Indeed, some papers show that group-housing is bad for murine welfare.

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

We wish to study hippocampal-dependent functions such as spatial memory, memory for individual members of one’s species, and anxiety. Animals that have been terminally anaesthetised can be used for some very simple preliminary physiological work, but cannot be used to test real-life, awake behaviour by definition. The link between physiology and behaviour would be missing.

Modelling learning in flies, fish, and worms is of potential interest, but cannot hope to model learning in humans. The hippocampal formation in terms of anatomy and physiology is well conserved across most mammals (except Cetaceans such as dolphins and whales) and is strikingly similar in rodents, non-human primates and humans. We are not just interested in learning per se, but mechanisms of learning and memory as applied to humans.

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

The techniques contained in the protocols below have been refined over 50 years in London by the previous supervisor of the current Licencee, and in the last 25 years by the current Licencee.

Minimisation of pain, distress, and lasting harm has been an ever-present goal throughout this period. Scientific visitors are often surprised at how our animals appear unruffled by the procedures. Implanted rats and mice behave very freely, running around, peering over edges, rearing, almost identically to non-implanted rats and nice.

Minimising pain, distress, & lasting harm General points and proxy social housing

The animals get opportunities to experience varied environments and to behave spontaneously and to exercise. Each animal is kept in a cage which meets the guidelines of the Code of Practice and contains wooden blocks, paper for nest material, and/or other objects. Rats and non-aggressive mice are housed in pairs or larger groups until surgery.

We have piloted and are now using 'proxy social housing', even after implantation. This involves side- by-side housing using mesh partitions within large cages (i.e. such that each side still greatly exceeds the minimum required floorspace for a single animal).

Specific Protocol

steps Step re Handlin

All protocols involve extensive handling of rats, which we find minimises distress, rats habituating to experimenters/staff very quickly. We cuddle and tickle rats, which greatly reduces their stress, as has been shown in published work.

Responses in mice to handling are more strain-specific, but most appear to habituate less to humans. Accordingly, mice are handled less than rats, and we have procedures

involving less human handling. We typically allow the mouse to spontaneously walk onto a surface such as a cage lid or plate, then transport that surface with the mouse on it, and place the surface in the testing environment, and then allowhe mouse to walk off the surface into the environment.

In general, of course, we use non-tail handling methods in both species except in rare circumstances.

Step re Food Deprivation

There is abundant long-established evidence that chronic mild food restriction is beneficial to rodents, just as it is for non-human primates and humans. The following is a small selection and focused on animals only, particularly rats and mice.

Weindruch, R. and Walford, R.L., 1982. Dietary restriction in mice beginning at 1 year of age: effect on life-span and spontaneous cancer incidence. Science, 215(4538), pp.1415-1418

Weindruch, R., Walford, R.L., Fligiel, S. and Guthrie, D., 1986. The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake. The Journal of nutrition, 116(4), pp.641-654.

Masoro, E.J., 2000. Caloric restriction and aging: an update. Experimental gerontology, 35(3), pp.299- 305.

Anderson, R.M., Shanmuganayagam, D. and Weindruch, R., 2009. Caloric restriction and aging: studies in mice and monkeys. Toxicologic pathology, 37(1), pp.47-51.

Colman, R.J., Anderson, R.M., Johnson, S.C., Kastman, E.K., Kosmatka, K.J., Beasley, T.M., Allison, D.B., Cruzen, C., Simmons, H.A., Kemnitz, J.W. and Weindruch, R., 2009. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science, 325(5937), pp.201-204.

Niemann, B., Chen, Y., Issa, H., Silber, R.E. and Rohrbach, S., 2010. Caloric restriction delays cardiac ageing in rats: role of mitochondria. Cardiovascular research, 88(2), pp.267-276.

The level of restriction in the present licence is mild (milder than for most experimental groups in the above-cited studies): we only deprive to 85-90% of free-feeding bodyweight.

Step re Behavioural training

Most of the behavioural testing procedures use appetitive reward and the animals readily and eagerly participate in them.

Step re Surgical procedures

We have introduced several changes over the years to improve surgical techniques, and continue to improve. These include:

a) modelling surgeries extensively in advance, e.g. using 3-D sketchup software, and very thoroughly- cleaned cadaver skull, so as to reduce surgery time

b) Additional assistant during surgery (typically a highly experienced Licensed technician) for all surgeries to ensure sterile procedures and monitor anaesthesia

c) more comprehensive aseptic technique

d) refined instrument handling and layout procedures

e) drilling at lower speed, applying sterile saline as a coolant

f) superglue screwsites

The animals are monitored carefully post-operatively to make sure they remain healthy and recover quickly. The recovery period, while typically 7-10 days, is tailored to each individual animal, and can be longer (rarely required.)

Step re Electrode lowering into target area/drug delivery/recording during behavioural tasks

Both rats and mice rapidly learn to accept the light restraint in a cotton towel that is usually necessary for fixing the headstage amplifier connection to the electrode drive. In rats, this is very rapid, most show high levels of learning within one day, producing minimal resistance the next day. In mice, this can take longer, up to 3/4 days. This rapid habituation occurs because the animals learn that despite restraint, there is no aversive outcome.

We have increased and keep increasing the number of electrodes per animal using improved technology, so that richer insights may be gained from a single animal. (Subsequently, therefore, we use fewer animals.)

Step with Termination of experiment with perfusion

The chest cavity will only be opened once it has been ascertained that the animal is deeply anaesthetised using at least two of the following tests: 1) Loss of acoustic startle; 2) Pedal pinch reflex;3)Corneal reflex.

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

Our electrophysiological techniques are quite specialised, so often refinements come by constantly reading e.g. publications such as Barkus et al (2022), and acting as a reviewer for, peer-reviewed methodologies for the different experimental procedures.

General, cross-disciplinary procedures such as of surgery, husbandry, handling, come publications such as:

- Code of Practice for Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes
- LASA Guidelines
- RSPCA Animals in Science guidelines
- UFAW Guidelines and Publications

- NC3R’s and Procedures with Care
- ARRIVE and PREPARE guidelines

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

We are constantly reviewing best practice in our scientific studies, including regular discussions with other scientists both within the university and at other institutions. This involves not just classic cross- discipline improvements in welfare, husbandry, and handling, but in housing and testing enviroments, and recording technologies that increase the 'insights-to-suffering' ratio.

Additionally, the local AWERB, NIO, NACWO, NTCO and NVS regularly inform, and disseminate information regarding reduction, replacement and refinement, including new publications of guideline and research articles, and presentations and reports from collaborators, peers, and animal welfare bodies. I attend courses, and read.

General, cross-disciplinary procedures such as of surgery, husbandry, handling, come from publications such as:

- Code of Practice for Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes
- LASA Guidelines
- RSPCA Animals in Science guidelines
- UFAW Guidelines and Publications
- NC3R’s and Procedures with Care
- ARRIVE and PREPARE guidelines

It is important to note that our electrophysiological techniques are quite specialised, so often refinements come by constantly reading, and acting as a reviewer for, peer-reviewed methodologies for the different experimental procedures.

Finally, I 'follow' organisations such as LASA, Norecepa, the National Centre for the 3Rs, on Twitter.

28. Mechanisms of Immune Cell Migration and Activation in Young and Aged Inflamed Tissues

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Inflammation, Leukocytes, Vascular Biology, Ageing, Immunology

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of the project will be to elucidate the molecular basis of immune cell recruitment and activation in physiological and pathological settings and in inflamed aged tissues.

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

Why is it important to undertake this work?

Acute and chronic inflammatory reactions, particularly in the ageing population, are an increasing financial burden on the healthcare system. As movement of white blood cells in the body and their immune functions play a critical role in driving inflammatory disorders, understanding white blood cell behaviours in inflammatory conditions requires further investigations. Our strong basic research programme with a clear translational element, has to date, made seminal contributions to the field of inflammation through an increased understanding of its key components. These include generating greater insight to the molecular and cellular changes that occur in inflamed tissues, such as in blood vessels,

and changes in the movement and activation of white blood cells. Crucially, our findings have shed light on these events in inflamed aged tissues. Thus, the major benefit of our work is knowledge creation of fundamental pathophysiological processes that mediate inflammatory diseases, and how such responses become aberrant with age. Importantly, our findings have already identified numerous novel pathways that could be targeted for treatment of systemic inflammatory conditions and ageing- associated diseases, validating the strength of our objectives and methods

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

Increased understanding of immune cell migration: Neutrophils are a key component of innate immunity and are the first immune cells to arrive at sites of inflammation. Building on our previous works, the proposed studies aim to extend our understanding of neutrophil interactions with different components of blood vessel walls and how these interactions are regulated by tissue resident cells (e.g. macrophages and mast cells) that reside in close proximity to the outside of blood vessels. As well as investigating these fundamental events in physiological reactions (i.e., acute and resolving inflammation), we will do likewise in pathological models such as those induced by ischemia- reperfusion (IR) injury, particularly in the context of ageing. These works will identify how neutrophil behaviour is dysregulated in pathological scenarios and hence could identify cellular and molecular pathways that may be amenable to therapeutic interventions.

Characterisation and analysis of abnormal modes of neutrophil breaching of venular walls (TEM): Our laboratory has discovered many abnormal modes of neutrophil behaviour when they interact with blood vessel walls. This includes a response whereby neutrophils pause as they are migrating through blood vessel walls to gain access to the tissue, and instead return to the blood circulation. We have termed this neutrophil response, neutrophil reverse transendothelial migration (rTEM), a behaviour that we have associated with the spread of local inflammation to other organs that are distant from the initial inflammatory site. We will aim to gain further insight into the nature of these

rTEM neutrophils and how they spread to distant organs such as the lungs and if they are responsible for causing distant organ damage. Furthermore, we will aim to address the crucial question of whether neutrophil rTEM plays a normal physiological role, e.g. to provide greater intravascular immunity.
Works conducted on animal models will be extended to human studies through collaborations with clinical colleagues to help identify neutrophils with similar molecular characteristics.

Increased understanding of how blood vessel leakage may influence neutrophil trafficking: We propose to acquire greater understanding of how blood vessel leakage (a key feature of acute inflammation) can regulate the generation of rTEM neutrophils. As increased blood vessel leakage is a common problem in older people, we will conduct experiments in both young and aged mice to compare the different responses in these 2 age groups. The findings of this work will address the exciting new hypothesis that targeting local blood vessel leakage may be a potential treatment for suppression of remote organ injury, such as acute lung damage, which is a common complication in elderly patients.

Increased understanding of the impact of age on inflammatory responses: We propose to acquire greater understanding of how old age influences neutrophil and blood vessel responses, leading to local and distant organ damage. This knowledge will be of profound

importance in understanding the impact and mechanisms through which ageing dysregulates inflammatory responses to cause life threatening diseases such as acute respiratory distress syndrome (ARDS; a severe type of pneumonia) and may contribute to better clinical management of ageing-associated disorders.

Investigations into the bridging of innate and adaptive immunity by neutrophils: We have previously demonstrated that acute inflammation leads to the accumulation of neutrophils within the lymphatic system, where they interact with cells of the adaptive immune system e.g. lymphocytes. We now propose to extend these findings to investigate the role of this response in bridging innate and adaptive immunity during acute inflammation such as that induced by ischemia/reperfusion (IR) injury. This work is anticipated to identify better clinical strategies aimed at controlling abnormal activation of the adaptive immune system that is believed to cause many inflammatory and autoimmune disorders.

In addition to the specific scientific goals listed above, as our proposed works will involve use of advanced imaging methods, we believe a key benefit of our work will be the establishment of novel microscopy methods, as per our strong track record and high international standing in this area.
Specifically, we will continue to develop and refine our intravital microscopy (IVM) methods to acquire maximal information from a single in vivo experiment. Publishing details of these models will likely lead to the design and generation of novel genetically altered (GA) mice that will be of value to the wider scientific community.

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

Findings of this work will be made available to the scientific community through in the short-term via presentations at national and international meetings (first 1-3 years). Beyond this, the ultimate goal will be to publish the findings of our works in reputable scientific journals in an open access format. Our strong publication record (16 peer-reviewed articles published under the existing license to date) and frequent participation at prestigious national and international conferences (e.g. >50 invited talks since 2018) endorse our ability to achieve this goal. We anticipate that the data generated from our work will be of interest to basic scientists and clinicians in academia and researchers in the private sector. Our findings will enhance knowledge of fundamental disease mechanisms, identify novel molecular pathways that could be targeted for future drug developments and open new scientific avenues for exploration of inflammatory mechanisms.

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

In line with the rigorous policies of our funding bodies on data sharing, and our University’s policy on data management, we consider it an ethical responsibility to ensure data generated, and in particular data derived from animal studies, is openly accessible. As such, after a limited and appropriate period of exclusive access to data in order to prepare research manuscripts, all relevant raw data will be shared freely and in a timely manner with the wider research community. All data submitted for publication will be from optimised, validated, multiple repeat experiments, to pass rigorous review standards for peer-reviewed journals and will be supported with appropriately detailed methods, outlining acquisition and analysis for reproducibility.

Hence, fundamentally, our primary mechanism to disseminate research findings will be through open- access publication in peer-reviewed journals, supported and promoted by

review articles, local and international conference presentations, and public-engagement activities. Publications (involving successful or unsuccessful methods) will be made publicly available via PubMed, and novel findings are promoted on social media platforms such as Twitter. Furthermore, our regular participation in scientific conferences will continue to offer a valuable platform to showcase our work and establish collaborations. With respect to the latter, our work to date involves >20 international and national collaborators, works that often involve validation of novel pathways and/or reagents for development towards new anti-inflammatory strategies.

As stated above, a key deliverable of our work is the generation of new tools and methods that we readily share with the scientific community. As an example, during the current license we developed a unique antibody to detect mouse Progerin, the specificity of which was validated using one of our genetically altered (GA) mouse strains. This antibody is now recognised by the scientific community as the most specific, and hence, most trusted anti-mouse Progerin Ab, and as such has to date been shared on request with numerous international experts specialising in ageing research. Our collaborative works with this novel reagent has significantly enhanced research into the mechanisms and therapeutic treatment of the rare and 100% fatal disease Hutchinson-Guilford Progeria Syndrome (HGPS).

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.

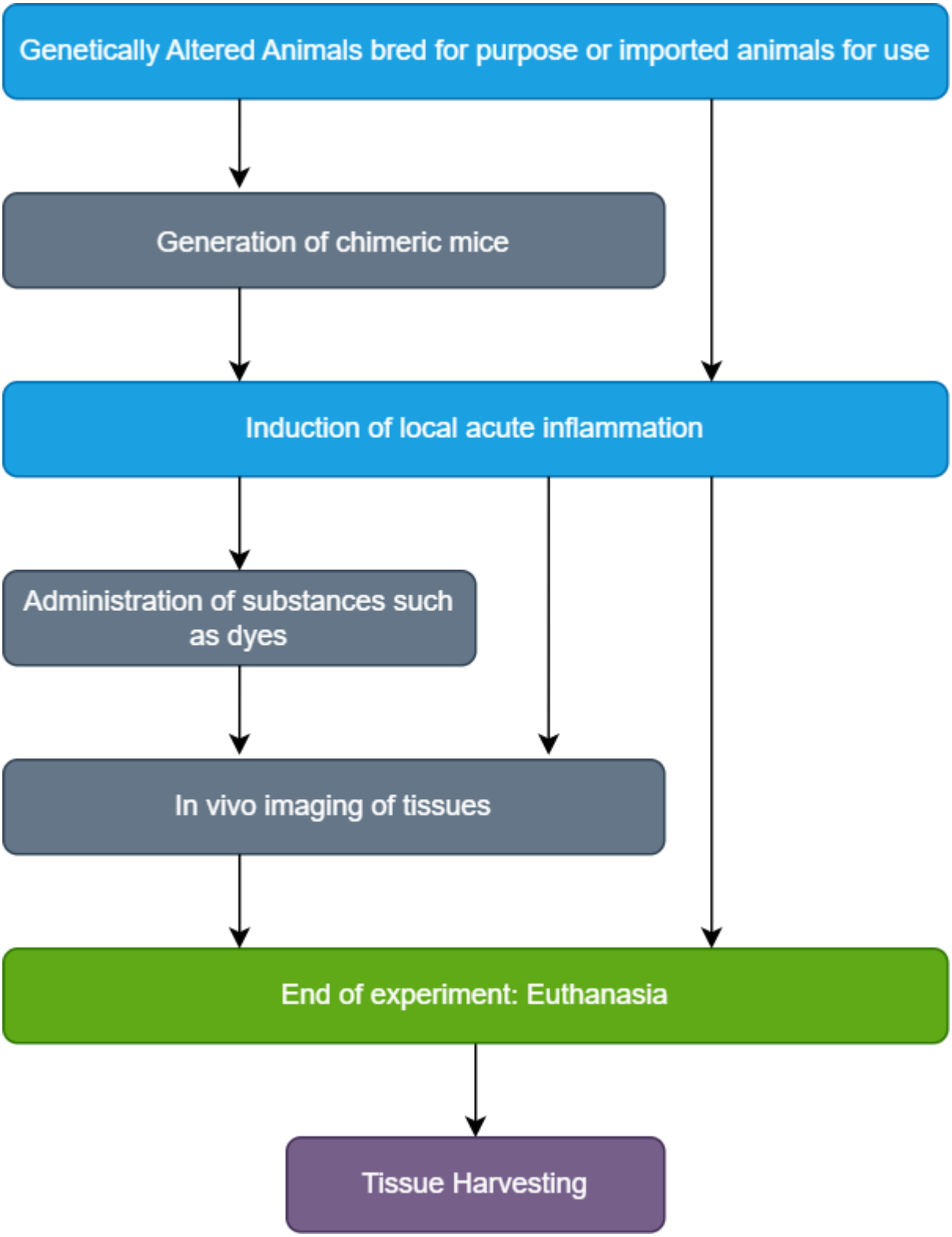
Explain why you are using these types of animals and your choice of life stages.

Fundamental parts of the proposal necessitate an integrative approach where leukocyte behaviour will be assessed in their microenvironments, such work requiring the use of in vivo models. Additionally, as the scientific community widely uses mouse models, they are associated with an extensive catalogue of scientific tools: Many genetically altered (GA) mice allowing specific deletion or overexpression of genes, selective and thoroughly validated anti-mouse antibodies and recombinant mediators of inflammation. For our work, we will only use adult (from 7 weeks old) wildtype and GA mice with no notable adverse phenotypes (e.g. exhibit normal breeding, weight and behaviour). Experiments will also involve the use of aged (>15 months) mice, that are considered ‘old’ and thus represent the most suitable model for ageing research. The latter constitutes a significant component of our current and on-going research and extends our recently published works on this topic.

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

The work uses well-established models of inflammation to study leukocyte interactions within the vasculature, that enable the assessment of novel inflammatory mechanisms. These models include simple and well-controlled in vivo models (e.g. cremaster muscle inflammation) with more complex models (e.g. lung inflammation), providing a direct link to human biology.

Stages of a typical experiment is presented in the flow chart below:



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

Some GA lines (where ‘global’ deletion/modification of the gene of interest is known to be embryonically lethal or cause a harmful postnatal phenotype) will be conditionally or inducibly expressed and used in conjunction with appropriate care/welfare programs that will be put in place on the advice of our Named Veterinary Surgeon (NVS). Thus, although we are not anticipating any adverse effects due to the use of new inducible/conditional mouse models, such possibilities will be monitored (e.g. regularly assess the breeding programme, weight and behaviour of the litters).

We will occasionally generate chimeric mice, which requires sub-lethal irradiation of recipient mice to deplete their hematopoietic stem cell compartments, followed by reconstitution of bone marrow (BM)/myeloid progenitor cells by intravenous injection of BM cells from a donor mouse. This procedure can lead to the need for euthanising a small percentage of mice (~2-5%) during the first week of the protocol (whilst the mice have low levels of white blood cells and are therefore susceptible to infections).

Most of our experiments will involve administration of substances under general anaesthesia resulting in animals experiencing mild or transient discomfort. Analgesic will be provided if required and monitored all throughout the experiment. No lasting harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal, oral gavage) are expected. Animals under non-recovery general anaesthesia may experience mild distress during the administration of the anaesthetics and analgesics only, but no pain.

Some of our work will require methods that cause non-surgical short-term and reversible restriction of normal blood flow to one or more limbs, ears or testes under general anaesthesia. Where animals are allowed to recover for the limited period specified in the protocols, particular care will be given to ensure minimum discomfort to the animal.

As the study of ageing is an important facet of our scientific research, we will implement special monitoring and care requirements (e.g. daily monitoring for general well-being, accessibility of flood/fluids with wet-mash diet) as well as devised specific humane endpoints for these aged animals that are detailed in the protocols below.

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

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

Non-recovery: 25%

Mild: 50%

Moderate: 25%

Severe: 0%

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

- Killed

Replacement

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

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

Whilst ex vivo/in vitro works are undoubtedly important and valuable, in vivo models are essential so that inflammatory responses and their amelioration using pharmacologically active molecules can be investigated within physiologically relevant scenarios (e.g. a vascularised tissue) and in their totality, incorporating key elements of inflammation e.g. distinct leukocyte subtypes, ECs, pericytes, perivascular cells, physiologically relevant shear, lymphatic vasculature and the complex extracellular milieu that is currently impossible to accurately model in vitro. Furthermore, as the scientific community widely uses mouse models, they are associated with an extensive catalogue of scientific tools: Many GA animals allowing specific deletion or overexpression of genes, selective and thoroughly validated anti-mouse antibodies and recombinant mouse cytokines.

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

We have extensive expertise in the use of in vitro models for investigating leukocyte and vascular responses and as such in many instances these will comprise our first line of study. For example, many pilot studies will be undertaken primarily using in vitro approaches with cell lines and whenever possible, using human cells to link the findings to human physiology and pathology. We are continually developing more advanced in vitro systems, such as in vitro models of transient hypoxia followed by re-oxygenation to mimic conditions of ischemia-reperfusion injury. Another part of our work is in close collaboration with a research group specialising in genetic screening of fruit fly models to identify gene candidates relevant for our mouse models.

Why were they not suitable?

Our studies involve many experiments conducted in test-tubes using cultured cells, which help us to understand how individual cells function. However, the findings from these studies ultimately need to be evaluated within the complete physiological setting offered by animal systems. As stated above, this is because we cannot accurately recreate in the laboratory, complex and diverse structures that are critical to the occurrence of inflammation. These include diverse blood vasculatures, tissues/organs and multiple cell types organised in a unique network that is not possible to reproduce in vitro. Hence, to acquire physiologically and pathologically meaningful findings, it is necessary to employ animals. We will actively seek, review and incorporate wherever feasible, alternative means of non-regulated experimentation throughout the duration of the project e.g. sophisticated and relevant in vitro co-culture 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 use of complex co-culture in vitro models (as detailed above) has led to significant reduction in the use of animals. In addition, our extensive experience with in vivo models enables us to plan and conduct experiments such that the minimum number of animals are used with the minimum level of procedural severity. The number of animals estimated for this project include the necessary control groups for comparative and quantitative analyses. Typically, we need group sizes of 6-8 to achieve the quality of results we need. This is based on the applicant's extensive experience, as supported by discussions with local statisticians, collectively aiming to achieve a minimal statistical significance level of 0.05%. Original data generated within our team commonly supports multiple parallel projects, thus reducing the number of animals used. We regularly refine our in vivo imaging methods with the aim of yielding better quality, more in-depth and overall greater quantity of data. This means we also routinely quantify multiple parameters in the same animal, ensuring maximal output. By working as a team, experiments are designed such that maximal information is obtained from every experiment by multiple researchers. This ensures the use of fewer animals as well as ensuring a more coherent mode of experimentation and overall maximal productivity.

Procedures involving surgical steps, e.g. surgical exteriorisation of tissues, may require sham surgical groups to control for the impact of tissue handling on the parameters of interest. Projects involving new quantification methods (e.g. measuring neutrophil clustering in the lungs), will require optimization to validate the optimal time-points for subsequent processing of the tissues and imaging prior to image analysis. These will be kept to an absolute minimum and will be dropped if considered un-necessary beyond a certain stage of the project. 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 tests will be used as appropriate to compare experimental groups (guided as need be by the NC3Rs Experimental Design Assistant; EDA). Of importance, as all our work is original (i.e., novel hypotheses stemming from our own findings and addressed using unique imaging methods and GA animals), we are confident that there is no redundancy and/or unnecessary repetition of studies.
Furthermore, at the end of each protocol, several parameters, organs or tissues will be collected for post-mortem analysis to decipher the biological responses in vivo and 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?

We have and will continue to use efficient breeding programs to ensure production of mice with relevant genotypes whilst the ones with no value are kept to a minimum. Animals that are not utilised within the protocols of the project will be used for post-mortem analysis of

tissues, where valuable assay-based research can be conducted. At the termination of each experiment using an animal,

tissues will be collected in abundance for further analyses, either immediately or in the future where they may be kept in cold storage until required. Furthermore, original data generated within our team commonly supports multiple parallel projects, thus reducing the number of animals used. Where new pharmacological agents are being assessed, dosing regimens will be determined based on literature searches, advice of collaborators and/or pilot works. With respect to the latter, we will typically test two doses of the drug (administered locally or systemically, depending on whether we wish to target a specific tissue or circulating cells, respectively), usually involving no more than 3 mice/group. Doses will be adjusted when no effects, or rarely adverse effects, are observed.

Refinement

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

Which animal models and methods 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 seeks permission to conduct experiments on mice, these being the lowest vertebrate group on which well characterised and minimal severity inflammatory models have been developed. In addition, there are many valuable genetically altered strains of mice available as well as a wide range of inflammation inducing and inhibiting drugs that can be used in mice. Furthermore, all our procedures (e.g. cremaster muscle, skin, peritonitis and lung inflammation models) are well-established and classified as being mild or moderate in severity.

Most of our work is of short duration (2-4 h, rarely up to 6 h (>5%) and involves general anaesthesia, under which animals are unconscious. However, where minor procedures must be performed on conscious animals (peritoneal inflammation, administration of substances by oral gavage or injection of anaesthetic agents), we will routinely employ analgesia as required for pain reduction and/or employ sterile procedures to eliminate/minimise the occurrence of infections. Some of our work (e.g. in vivo imaging) will involve terminal analgesia/anaesthesia, conditions under which animals suffer no pain.

Environmental enrichment such as the addition of cardboard shelters, wooden gnawing blocks and increased amounts of nesting material to all IVC cages is standard practice within the animal unit. Such enrichment strategies designed to increase animal welfare will be continually reviewed and new practices implemented as and when deemed appropriate by the NVS/NACWO.

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

Non-mammalian animals are limited in their use because they do not recapitulate mammalian vascular microenvironments; and their organ anatomy and composition, such

as the nervous, cardiovascular or lymphoid systems, differ radically. Additionally, 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 physiologically relevant results. Furthermore, a more limited catalogue of scientific tools is available for non-mammalian species.
We will not use embryos or very young animals as their immune system is immature and cannot mount appropriate inflammatory reactions. A major component of this project investigates immune responses in aged mice (>15 months) and all other aspects of the project require adult mice, precluding the use of juvenile mice.

Several of our protocols involve the longitudinal analysis of immune and vascular responses in vivo in both local and distal organs following the induction of an acute inflammatory response in conjunction with, but not limited to, the administration of immunomodulatory substances. To minimize acute pain and stress, as well as potential side effects associated with long-term anaesthesia (e.g. blood pressure and temperature drop) animals will be typically subjected to short-term general anaesthesia with recovery.

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

Animals recovering from anaesthesia following a procedure, such as the non-invasive application of ischaemia inducing elastic bands, will first be monitored until they are fully conscious, and their general behaviour will be closely monitored by trained personnel to ensure that the animal appears pain-free and healthy and is able to eat and drink normally. If required, analgesics will be provided. Where mobility may be reduced, wet/dry diet will be provided on the cage floor for easy consumption. Animals undergoing terminal anaesthesia will have their reflexes checked periodically to ensure the animal does not regain consciousness and dosed as necessary to maintain anaesthesia.

Aged mice will be carefully monitored by staff trained to work with ageing animals e.g. NACWOs. When working with aged mice, to accommodate for loss of animals due to old age, group sizes will be increased and we will avoid single housing whenever possible. Longer drinking spouts will be used, and animals will be monitored for adverse effects such as changes in weight, dermatitis, piloerection, paleness, changes in mobility, lumps, eye defects, abnormal respiration, or stools. If these are observed, animals will be treated accordingly, and animals that likely to exceed the severity limit will be humanely killed.

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

PREPARE guidelines and NC3Rs up to date recommendations will both be considered in conjunction with advice from our local NVS and NACWO.

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, we’ve signed up to the NC3Rs newsletter, we will meet the NC3Rs Regional Programme Manager, and attend Regional 3Rs symposia.

29. Antibody 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
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Antibody, Monoclonal, Polyclonal, Diagnostic, Identification

Animal types	Life stages
Mice	adult
Rats	adult
Rabbits	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To produce antibodies in the blood and tissues of rodents and rabbits. In doing so we will support and help the advancement of biological knowledge and understanding, and the development of new 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?

Substances found in the immune system, such as antibodies can be used to show the presence or lack of biological material, to show the therapeutic effects of substances, or

may be a therapeutic agent in their own right. Antibodies may also be used in new imaging or diagnostic procedures. They will only be produced when there is no other method of achieving these goals. By centralising this service, we can refine the expertise available at one site, and remove the need to develop new techniques at many other sites.

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

We will supply customers with special types of materials derived from the immune system (e.g. monoclonal and polyclonal antibodies) for use in their own research and development projects. These customers may publish the results of their work or include them in talks and seminars.

In some instances, the material produced on this Licence will replace the need to use animals in other future experiments, as they will be used to establish animal alternatives, such as hybridomas cell lines and Phage Displays. These will then be published on open databases and made available for others to use.

In some instances, the material produced in this licence may be of therapeutic value for animal and human patients in treating or identifying and diagnosing diseases.

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

Monoclonal and polyclonal antibodies are used in diagnostic tests, imaging tests and may be therapeutic agents themselves. In the short term this will help researchers further their understanding of biological systems and mechanisms and aid the development of new medicines. When the monoclonal or polyclonal antibody is the medicine itself, they may attack and destroy tumour cells, they

may be used to help deliver medicines to specific target areas, for example a tumour, or attach to the target and label it, enabling another chemical to find it more readily. It may also help target the delivery of radioactive doses in the treatment of cancers. This will benefit human and animal patients whose treatments are reliant on these antibodies, when the antibodies are not available from other sources or methods of production.
In the medium to longer term the advancement of knowledge and treatments brought about by this work will help improve existing treatments and make possible new treatments to people and animals. This may be done by using these antibodies produced in this licence to label material in samples in order to show the presence or absence of other target materials of interest, or by helping experimental material to target the material of interest once it is labelled with the antibody produced in this Licence.

Whenever possible, the antibody's produced under this Licence will be used to establish invitro sources of that antibody, which can then in turn be used as a non-animal source of the antibody for future projects. This non-animal antibody source will then be advertised in libraries and brochures and made available to other interested parties. This will be of benefit to other scientific projects giving them ready access to non-animal sources of antibodies. As part of our pre-project screening questionnaire all customers will be asked to do this.

Prior to agreeing to carry out this work we require customers to complete a project request questionnaire which includes a request for the customer to provide evidence of the searches they have made to find alternative non-animal sources for the antibodies they

require and justify why they cannot produce these antibodies by using a non-animal technology. Once they have completed this work, we request that the non-animal source of antibodies is published and made available for others to use.

When new or more refined techniques are identified, these will be presented at the AWERB meeting and if appropriate presented as posters at meetings and symposia. Antibody sources will be shared on Library listings and company brochures to ensure the wider community can access them to support future work without the need to use animals to produce these antibodies in future.

By maintaining a highly skilled, dedicated team with knowledge of this procedure, we will collaborate with customers, performing assays to the highest standards. The maximum output will be achieved by using the minimum number of animals and by assessing the circulating antibody levels of the animals before collecting the monoclonal or polyclonal antibodies.

Some monoclonal antibodies are used in diagnostic test kits. This will help both human and animal patients receive accurate diagnosis.

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

By maintaining a highly skilled, dedicated team with knowledge of this procedure, we will collaborate with customers, performing assays to the highest standards. The maximum output will be achieved by using the minimum number of animals and by assessing the circulating antibody levels and ensuring that they are at a sufficiently high level before collecting the monoclonal or polyclonal antibodies.

When new or more refined techniques are identified, these will be presented at the AWERB meeting and if appropriate presented as posters at meetings and symposia. Antibody sources will be shared on

Library listings and company brochures to ensure the wider community can access them to support future work without the need to use animals to produce these antibodies in future.

While ensuring the severity of the procedure the animal experiences is not increased, whenever possible, animals will be given multiple antigens in one injection to reduce the overall number of animals used in the lifetime of this experiment. Multiple antigen immunisations will not be given if this would compromise the validity or quality of the antibodies produced. If the customer does not know whether multiple antigen immunisations are possible, then a pilot study may be run to establish their feasability.

Species and numbers of animals expected to be used

- Mice: 4000
- Rats: 1000
- Rabbits: 1600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 rabbits, rats and mice are the lowest vertebrate group in which antibodies can be produced in the volumes required. Mature animals are required as they will have developed their own immune system, giving them the ability to produce antibodies against other materials and proteins. Younger animals may not produce sufficient antibodies as they do not yet have their own fully developed immune system. In some cases, rabbit antibodies may be specifically required.

When working under the authority of this Licence, mice will always be the species of first choice, followed by rats, and only if these species are not suitable will rabbits be used. Of rabbits, rats and mice, mice are the species least sentient and still able to produce the antibodies required. When mice cannot be used rats may be used as an alternative. Only where rats and mice cannot be used will rabbits be used. Rabbit antibodies are also able to recognise the parts of foreign material of human antigens (epitopes) that do not stimulate an immune response in rodents. This enables us to increase the number of targetable epitopes and therefore facilitating the generation of antibodies that cross react with mouse orthologs of human antigens.

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

Animals will receive an injection of the material for which antibodies against it are required. This material may be suspended in a substance designed to optimise the animal's immune response against it (called an adjuvant). An adjuvant is used to modulate how the immune system responds to the material. In some cases, the adjuvant will slow the release, meaning less injections are required in the overall protocol. The material is normally administered by injection just under the skin (sub- cutaneously, SC), but when this route will not produce a strong enough immune response it may be into the skin (intra-dermally, ID), into a muscle (intramuscularly, IM - not mice) or directly into the blood stream in a vein (intravenously, IV) into the abdomen (Intraperitoneally, IP - not rabbits). This slowed release, produced by the adjuvant will prolong the animal's exposure to the substance, which will have the effect of producing as higher monoclonal or polyclonal antibody level as possible. Examples of adjuvants include but are not limited to; Complete Freund's adjuvant (CFA), Incomplete Freund's Adjuvant (IFA), mineral oils, Titre Max, Alum, squalane, in water emulsions, monophosphoryl lipid A (MPL), Ribi adjuvants, Montanides, polymeric microparticles, saponins, immunostimulatory nucleic acids, toll like receptor agonists, cationic liposome formulations (CAF), nano particles, EMULSIGENS, Specol and Syntex Adjuvant Formulation (SAF). All adjuvants used must first be approved for use by the local Animal Welfare and Ethical Review Body (AWERB)

Typically, animals will only suffer mild transient pain with no lasting harm from the administration of these substances by injection using standard routes (e.g. subcutaneous, intra-peritoneally (rodents only) and on rare occasions intra-muscularly or intravenously). Animals may experience mild transient discomfort during blood sampling. The injections may be repeated to ensure the circulating antibody level reaches its maximum level without causing unnecessary harm to the animal.

Animals will be humanely killed and then the blood and tissues taken.

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

1. It is expected that the animals will look and behave normally. The Genetically Altered (GA) Rodents which may be utilised on immunisation protocols, are expected to be phenotypically sub-threshold.

2. Animals will be checked daily, and adverse effects recorded. Experienced handling of animals and instruments will reduce the risks to a minimum. Effects of significant departure from expected growth rates or other deterioration in condition will be mitigated through augmented nutrition or specific veterinary treatments where possible.

3. Blood samples will be taken from superficial blood vessels with no adverse effect expected. The maximum volume of blood removed during a bleed from which the animals will be allowed to recover, will be up to 10% in 24 hrs, or 15% of the estimated circulating blood volume within a 28-day period.

4. Any substances given are not expected to cause adverse effects at the doses given.

5. Injections carry a risk of local inflammatory reactions, but this will be reduced or eliminated through the use of aseptic techniques for inoculation and keeping the inoculation to the minimum volume compatible with the scientific objectives and proven competence in administration technique.

6. Fighting - animals suffering from serious wounds, showing other signs of distress or found to be in poor clinical condition will be immediately humanely euthanized by a Schedule 1 technique. In preference animals will be socially housed, but in the cases where they are socially incompatible, or display anti-social and aggressive behaviour, as indicated by injuries due to fighting, such animals will be singly housed, and superficial injuries treated under the guidance of the Named Veterinary Surgeon (NVS). Animals suffering from more serious wounds or showing other signs of distress will be humanely killed by a Schedule 1 technique.

7. Identification and tissue sampling methods may cause brief discomfort. No action expected to be needed.

8. In the case that adjuvants are required, careful consideration of the type of adjuvant and the appropriate route of administration will be made. No adjuvant will be used via the intravenous route and the intra-dermal route will be avoided. Complete Freund's Adjuvant (CFA) will not be used on more than one occasion in the same animal.

9. Animals with clinical signs of toxicity following injection that are anticipated to exceed a mild severity limit will be humanely killed. Indicative clinical signs of toxicity may be as follows: reduced food intake, acute weight loss, decline in general condition, pallor, increased isolation from cage mates, loose faeces, impaired gait and jaundice.

10. In less than 5% of animals, injection site reactions may be evident, and if they do occur will be reported and monitored, but these are expected to be minimal e.g. granuloma formation. Animals which have undergone a CFA immunisation at the beginning of the

protocol, have a small risk of developing lesions/ulceration (which will look like a sore) at the site of CFA Subcutaneous injection within a few days. Animals will be given appropriate veterinary care if required and if not resolved, will be humanely killed by a Schedule 1 method.

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

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

1. Blood samples will be taken from superficial blood vessels with no adverse effect expected. The maximum volume of blood removed during a bleed from which the animals will be allowed to recover, will be up to 10% in 24 hrs, or 15% of the estimated circulating blood volume within a 28-day period. This will be mild in 100% of the animals.

2. Any substances given are not expected to cause adverse effects at the doses given. This will be mild in 100% of the animals.

3. Injections carry a risk of local inflammatory lesions, but this will be reduced or eliminated through the use of aseptic techniques for inoculation, keeping the inoculation to the minimum volume compatible with the scientific objectives and proven competence in administration technique. Injections should be of a mild category in 99% of cases. The remaining 1% cannot be predicted. If the increase in severity cannot be immediately alleviated, then the animal will be killed by a Schedule 1 method.

4. Fighting (<1% cases) - animals suffering from serious wounds, showing other signs of distress or found to be in poor clinical condition will be immediately humanely euthanised by a Schedule 1 technique. In the case of rodents that display aggressive behaviour, as indicated by injuries due to fighting, such animals will be singly housed whenever possible and superficial injuries treated under the guidance of the Named Veterinary Surgeon. Animals suffering from more serious wounds or showing other signs of distress will be humanely killed by a Schedule 1 technique.

5. Identification and tissue sampling methods may cause brief discomfort. No action expected to be needed.

6. In the case that adjuvants are required, careful consideration of the type of adjuvant and the appropriate route of administration will be made. No adjuvant will be used via the intravenous route and the intra-dermal route will be avoided. CFA will not be used on more than one occasion in the same animal.

7. Mice, rats and rabbits with clinical signs (<1%) following injection that are anticipated to exceed a mild severity limit will be humanely killed. Indicative clinical signs may be as follows: reduced food intake, acute weight loss, decline in general condition, pallor, increased isolation from cage mates, loose faeces, impaired gait and jaundice.

8. Injection site reactions will be reported and monitored, but are expected to be minimal e.g. granuloma formation <5%. Animals which have undergone a CFA immunisation at the beginning of the protocol, have a small risk of developing lesions/ulceration (which will look like a sore) at the site of CFA injection within a few days.

Animals will be given appropriate veterinary care if required and if not responding to veterinary treatment within 72 hours will be humanely killed by a Schedule 1 method.

9. In some cases following SC injection some of the substance may leak into the skin layers causing irritation. Should this happen veterinary advice will be sought, and if the animal does not show signs of improvement within 72 hours it will be humanely killed by a Schedule 1 method.

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?

Monoclonal and polyclonal antibody cell cultures are used wherever possible, but these often only have a limited life span, and after a number of cell divisions will no longer produce the cells required. This project may be used to produce the new cells to establish the non-animal cell cultures.

Hybridomas are cells produced by the fusion between the B cells of an immunised animal and a tumour-like cell. By adding the tumour-cell, the B cell is now able to continue dividing infinitely and reproduce the antibody. This technique is used to generate stable clones of an antibody which can be used repeatedly after animal immunization. However, the genetic stability of the hybridomas is still an issue and antibody secretion can decrease over time; intellectual property rights limit access to some of the more genetically stable and reliable hybridomas.

For some antibodies, Phage Display may be a suitable In-Vitro alternative to In-Vivo antibody production, but not all cases.

Phage Display is a technology for the development and production of non-animal-derived antibodies. It involves inserting the genetic sequences of a repertoire of antibodies, originally sourced from a human or animal, or designed in silico, into the DNA of a virus that solely infects bacteria, known as a bacteriophage. Antibodies are thereby produced on the surface of the bacteriophage, and those bacteriophages bearing antibodies that interact with a specific target molecule can be isolated from a large collection of bacteriophages. Thus, the DNA encoding antibodies binding to the target of interest can be isolated. However, achieving a very high target affinity is an important aspect of antibody production and phage display technology binders have been found to have lower affinity, specificity and immunogenicity issues. Intellectual property rights limit access to some of the phage display technologies.

Aptamers are short sequences of artificial DNA, RNA, XNA or peptide that can bind to a specific molecule, or family of target molecules. They can exhibit both strong and weaker affinities, with some variable levels of off target binding. Sometimes these are described as chemical antibodies. Due to their variable levels of response, they are not always suitable

for all types of work. Only if they are not suitable, will work be carried out under the authority of this Licence.

B-Cell clones may be used where they secrete antibodies to a pre-determined antigen. Where they are not available to produce the required antibodies, this licence may be used to produce them, and may also establish b-cell clone cell lines which can replace the use of animals in future experiments.

Where In-Vitro techniques are not suitable, this Licence may be used to produce the required antibodies.

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

The monoclonal and polyclonal antibody producing cell lines only have a defined life span, and then need replacing. We have not been able to identify a non-animal alternative to establish the new lines to provide the antibodies required.

Antibody libraries, Phage assays and non-animal monoclonal antibody lists will be searched before any live-animal work is considered by the AWERB. The results of this search will be recorded in our pre-project questionnaire completed by the customer.

Why were they not suitable?

Non-Animal alternatives are not suitable as they do not exist for all materials. This project will also help to establish cell lines that produce the monoclonal and polyclonal antibody producing cells to replace further animal experiments.

Some commercially available antibodies may be extremely specific to an antigen, and so although they are very similar to those required, they may still not be suitable for the client research 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?

These numbers have been calculated taking historical business needs, the number of animals required to produce a suitable amount of monoclonal and polyclonal antibodies, and then multiplied out to support all forecasted customer demand for rat and mouse or rabbit projects over 5 years.

With the development of more humanised rodent models, and following discussion with our clients, it is anticipated this will bring a small rise in the number of rodent models used in the lifespan of this project.

Rabbit numbers have been calculated by considering the numbers of animals a prospective customer is currently using in projects in other countries, and then how many animals will be used on this project, and to establish the future non-animal sources of antibodies. It is no longer viable to continue outsourcing these rabbit projects overseas, as the tissues and materials are not always viable when received by the UK laboratory due to the long transport time.

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

We have looked at the numbers of animals used in previous similar experiments that were successful. This was then reviewed to ensure each experiment uses the minimum number of animals required to produce the required volume of immunologically derived material that will then be used for In-Vitro experiments or to establish cell lines and processes that will support future non-animal work using those monoclonal and polyclonal antibodies or aptamers.

Under this Service Licence it is our responsibility to ensure that customers have given appropriate consideration to the use of non-sentient alternatives and to identifying the most appropriate reduction strategies for research work. This will be achieved through a pre-project questionnaire, which is completed by the client and reviewed by members of the AWERB. Also, by the regular assessment of web resources (for example http://www.frame.or.uk and http://www.nc3rs.or.uk/), review of relevant journals, client meetings, attendance at industry meetings and symposia (for example LASA) and through the review of internal data.

The use of rabbits, rats and mice is preferred over other larger species that could tolerate more multiple samples as they will produce the sufficient volumes of antibodies and materials, and the volumes produced on larger species would be too great for our experiments, and greater than the volumes required to establish the non-animal cell lines for future use.

Wherever possible, and when it will not increase the severity of the protocol, animals will be challenged with multiple antigens, thus reducing the overall number of animals required to produce the full range of antibodies required. However, this will not be possible when the antibodies may cross react with each other reducing their viability, potency and usefulness.

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

Previous experience tells us the number of animals required to produce specific volumes of the immune cells that would be required to establish suitable amounts of monoclonal and polyclonal antibodies. In some cases, using genetically altered animals will enable us to reduce the amount of antibodies required, and so reduce the numbers of animals that are required for live experiments.

Refinement

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

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

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

Mice, rats and rabbits will be injected with very small amounts of the material of interest, and their bodies will react with an immune response against this material and produce antibodies. When the level of antibody in the blood is at an optimum level the animal will be humanely killed and the maximum possible volume of its blood taken. This way, the animal will not be kept alive for any longer than is necessary to obtain the immune cells and materials required for the subsequent In-Vitro work. Rabbits, rats and mice will produce the required volume of material, larger species will produce more material than is required, which would not be optimum.

These methods have been refined to avoid a painful injection into the muscle which can cause lameness, or skin which can cause skin ulcerations.

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

Rats, mice and rabbits will produce the types of immune cell (antibody) required in the volumes required to carry out later non-animal studies. Other lower species do not, or will not, produce sufficient volumes of the antibody. Also, lower species will not produce antibodies or produce sufficient quantity of antibody for the subsequent In-Vitro studies that are carried out using these cells. It is not possible to perform a quick experiment on terminally anaesthetised animals, as to produce the optimum antibody response in the host animal requires multiple exposures to the material of interest, over a longer period of time.

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

Animals will be given at least 1 week to acclimatise to their new environment once they have been received in their experimental area (see Obernier and Baldwin, 2006). Wherever possible both male and female animals will be used in the experiments. If only 1 sex of animal is to be used, then this must be justified by the requester before the work will be considered.

Animals will be weighed before the immunisation program commences, and future weights will be compared to this reference weight to assist with determining the welfare impact the procedures are having on the animal. The injections are not expected to cause any more than a mild transient pain as would be caused by a simple injection, and after the procedure the animals will be monitored for signs of adverse effects or reactions to the injection. The procedures are only carried out by experienced members of the team who have been trained in recognising the adverse effects or reactions expressed by an animal, such as changes to its normal behaviour or physiology.

Projects using Complete Freunds Adjuvant (CFA) must specifically state so at the pre-project questionnaire stage, and state why alternative adjuvants will not be a suitable

alternative or produce the same overall outcomes. Where the use of CFA over alternative adjuvants cannot be justified, small pilot studies may initially be undertaken to establish whether other adjuvants may be suitable alternatives.

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

Guidance on the operation of the Animals (Scientific Procedures) Act 1986.

LASA Good Practice Guidelines - Administration of Substances (Rat, Mouse, Guinea Pig, Rabbit). ARRIVE Guidelines.

Animal Welfare standards expected of Suppliers of Antibodies to Research Council establishments.

EURL ECVAM Recommendation on Non-Animal Derived antibodies.

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

I will regularly review current literature in order to keep up with current technologies in antibody production, both In-Vitro and In-Vivo, including information sources such as the NC3r's, NORINA and ECVAM.

30. Multi-tissue mechanics in the development and engineering of the posterior body axis

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

morphogenesis, differentiation, patterning, mechanics, stem cells

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?

Vertebrate embryos have a very similar layout of our major organs: a central nervous system, a segmented vertebral column and associates muscles and a central digestive tract. This 'body plan' is established early in development and is remarkably reproducible between individual embryos, and between species. This project aims to understand the principles underlying such robustness so that we can copy the same approach and improve the creation of tissues from embryonic stem cells in the petri dish.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 aims to reveal some fundamental principles about how cells make collective decisions during early development to become specific cell types. In healthy situations, cells will retain these specific cell types throughout an organism’s entire lifetime. However, in disease states such as cancer, cells reverse the programming received during development and re-enter a state that leads them to divide excessively, generate alternate

cell types, or migrate into the body and establish new tumours elsewhere. Therefore, the more that we understand the fundamental process involved in development, the better we can understand what goes wrong in cancer. Stem cells are cells that, like very early embryonic cells, can generate all cell types of the body. Recent work has enabled researchers to generate stem cells from cells of adults, that can then be redirected to many other different cell types. A major hope for the treatment of many diseases is through the use of stem cells to generate replacement cells, tissues or even organs. In addition, the ability to generate multiple cell types from an individual patient is revolutionising the study of disease and opening up the possibility of patient- specific medicine. For any of these possibilities to realise their full potential, we need to direct stem cells in a very well controlled way, without the risk of cells taking decisions that would be harmful such as entering a cancer-like cell state. By investigating the fundamental processes coordinating cell fate decisions and movement in early development, we hope to inform researchers working in the development of stem cell differentiation protocols and regenerative medicine. For this reason, we directly collaborate with stem cell researchers on a number of different projects and even have some in the lab.

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

We expect to gain a better understanding of how two key processes that operate during embryonic development work together. The first relates to cell movements, and the mechanical impact cell populations have on one another. The second relates to gene expression changes, and how signals alter this as cells move through the embryo. This will result in new conceptual advances that will impact the design of strategies to drive the differentiation of stem cells in culture. It will result in a series of publications in academic journals. We will ensure to communicate all outcomes of our work including both successful and non-successful approaches.

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

Short term: academics in a similar field will be informed of our research through conferences, pre- prints and peer-reviewed publications.

Mid term: academic researchers in the stem cell field will adopt new strategies for targeted cell differentiation

Long term: adoption of improved stem cell protocols in clinical and industrial settings.

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

We are active in the Open Research community, choosing to make all our results open to the community as fast as possible. We also seek to openly share our live imaging datasets with the community where possible. Any improvements in protocols will be rapidly communicated using forums such as F1000 and ZFIN.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 14400

Predicted harms

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

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

During the development of the embryo, cells have to decide on becoming all the different cell types that make up the adult body. They also have to do this in a very coordinated way, with tens of thousands of cells all deciding which part of the body they are going to generate. How this coordination is achieved is one of the great mysteries of biology. Our current understanding is based on the idea that long-range signals travel across the embryo to instruct cells about which cell type to become. Cells close to the source of the signal would receive a lot of it and therefore turn into one cell type, while those further away would receive less and therefore decide on a different fate. However, this only works in some cases, as the cells themselves are moving around very rapidly. Imagine a headmaster shouting instructions to an entire school of children playing outside. Whatever initial pattern that might be set up is going to be rapidly destroyed as they move around the playing field.

Another way is to make use of the fact that cells travel within distinct cell layers and are constrained by mechanical forces to move in streams. It's a bit like having the children back inside the school moving between different classrooms. Now the teacher can move through the corridors and give out specific instructions as the children move past. In the same way, specific signalling cells move through the embryo and pass on instructions to other cells as they move past. In reality, many teachers are required to walk the corridors of the school to make sure everything is kept in check. Note that in this model, it's the timing of when pupils meet the teachers that will determine how patterns form, rather than their spatial position as in the playground idea.

To understand this complex process, we collect information from each cell and its state (or each pupil and whether or not they have their shirt hanging out!) and track their movements as they move through the embryo. At the same time, we collect information about the instructions each cell is receiving. This is a huge amount of information that requires computer power to simulate the outcome. But once this is achieved, we can ask questions about what the minimal set of instructions are that are required to generate a well-coordinated pattern of cell differentiation.

Zebrafish embryos are ideal for this sort of large-scale experiment, for several reasons. Firstly, their embryos are visually transparent, meaning that we can image all the cells in the embryo at once.

Secondly, they develop externally outside of the mother and require only a simple temperature control for them to develop normally, meaning that we can culture them under a range of different microscopes and follow their development by time-lapse microscopy (a series of images taken of embryo development that allow for the generation of movies showing cells in action). Finally, we are able to insert new DNA sequences into their genomes that make fluorescent proteins which light up when certain genes are being switched on or off, allowing us to follow where specific signals are being received by cells as they travel through the embryo. Together, this means we can collect all the information required to watch how patterns in cell type differentiation occur in real-time, and across the whole embryo.

The outcome of this project is a better understanding of how normal development happens during the formation of the vertebrate body plan. Also, it provides new ideas about how we might control the differentiation and morphogenesis (the shaping of tissues and organs) with of stem cells cultured in the dish.

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

The adult zebrafish will be housed in a dedicated aquarium within the department, run by trained staff. We will generate genetically altered zebrafish by introducing modified genetic material at the 1 cell embryo stage and growing these embryos to adulthood. In order to know which fish contain genetic alterations we sometimes need to carry out genetic analysis via e.g. cutting a small portion of the fish's tail fin under general anaesthetic and analyse the genetic code inside this tissue. The fish is then kept in a separate tank with fresh water and the fin then regrows relatively quickly (within approximately 2 weeks). Once the fish have recovered from the anaestic they swim and behave normally, behaviour is monitored regularly by facility staff. Where appropriate, other methods of genotyping may be used, such as swabbing the surface layer of the skin. Adult fish will be maintained until a maximum of 30 months of age (although we aim to only keep adult fish until 18 months of age in the majority of cases). During this time, adult fish will be bred in specialised breeding tanks to enable the production of genetically altered zebrafish embryos. We very occasionally need to anaesthetise fish for the collection of eggs and sperm. At the end of the protocols fish will be humanely killed or supplied to other project licences or recognised establishments with the authority to breed and maintain genetically altered zebrafish of this type.

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

We do not usually expect there to be adverse effects to adults caused by the genetic alterations that we introduce. However, for example in the case of introducing optogenetic genes (a region of DNA that codes for proteins that can be activated and inactivated upon exposure to light of specific wavelengths), it is sometimes possible that some adverse effects might arise in the fish. If this occurs, we would expect such effects to be mild (such as thinner bodies). However, it is possible that moderate effects might occasionally arise (such as significantly bent body-axis, which might effect swimming). It is also possible that the survival of larvae to adulthood might not be as high in some genetically modified lines when compared to wild type lines. If moderate effects were to occur, we will humanely kill the affected fish. We do not expect there to be any adverse effects from breeding the zebrafish. It is unlikely but possible that fish might develop an infection following removal of a small part of the tail fin, in which case we will humanely kill the fish. For both genotyping and sperm/egg collection, it is possible that fish may not recover from anaesthesia but this is very unusual (less than 1%).

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

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

Zebrafish. Mild: 90%. Moderate: 10%

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 central aim of the project is to follow cells as they make decisions in their normal environment. Therefore, these experiments can only be performed in an animal. However, all experiments are performed at an early stage of life development (zebrafish embryos younger than 5 days old) which do not require protection in law.

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

We have introduced the use of embryonic organoids in our lab, derived from mouse embryonic stem cells. In addition, we make use of mathematical modelling to more rapidly explore mechanisms of development and better refine our hypotheses- that ultimately reduces animal use. Stem cell biology and regenerative medicine is currently undergoing a major shift towards the increased use of organoids- these are 3D multi-cellular aggregates that are allowed to grow and develop in a culture

dish (in vitro). While they can make many of the specific cell types of a multitude of different organs, the look and shape of these structures are usually very variable and often do not look like what is generated during normal development. We take the lessons learned from our understanding of multi- tissue development in zebrafish and use this to design new ways to better control and engineer the development of similar structures generated from mouse embryonic stem cells, called gastruloids.

As a rule of thumb, we like to let cells run their own, unmanipulated genetic programmes. We also let them talk to one another using signals that they normally would if they were in an embryo. Our central hypothesis is that by leaving these genetic and molecular components alone, we can refine development in a controlled way, simply by changing the external mechanical environment of the gastruloids. This would be like moving and shaping the school corridors, without changing the instructions shouted by teachers, or the ability of the pupils to listen to them. Instead, we act like a group of sheep-dogs corralling sheep into a tight group and guiding them to where they need to be.

However, to become effective sheep dogs, we need to know how this is achieved in normal development first and this is where our work with zebrafish embryos comes into play.

An additional way to replace a large number of animals for scientific research, is to make use of mathematical models and computer simulations to ask whether a certain set of observations are sufficient to generate the biological process we are interested in. This

helps us to define more precisely the experiments that are most scientifically interesting and of relevance to perform in the embryos on this project and will ultimately reduce the number of animals required for the project.

Why were they not suitable?

The central aim of the project is to understand how gene expression patterns are generated by cells as they go through the normal cell movements associated with embryonic development. While in vitro models of development can recapitulate multiple aspects of patterning, they do not acheive this with the same reproducibility and robustness that is seen in normal embryos. Our lab is set up to use zebrafish embryos where we can learn how this is achieved in normal development, and then transfer the lessons learned to mammalian embryonic organoids. Overall, it is a complementary approach.

Reduction

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

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

All of our experimental work will be carried out in zebrafish embryos younger than 5 days old (which are not protected under The Animals (Scientific Procedures) Act 1986. Animals older than 5 days old will only be used for establishing genetically altered zebrafish for subsequent breeding. The number of adult animals used is therefore solely related to the numbers required to maintain sufficient breeding stocks of animals.

Animal usages was based on requiring approximately 120 fish per new generation of fish for each genetically modified line. We will make a new generation for each line every year (5 generations per line). In addition, when generating new genetically modified lines, the F0 embryos need to be genotypically screened. Therefore, approximately 200 additional fish may be required per new line at the F0 stage to find appropriate founders to generate the F1 generation. Based on raising 15 established lines and generating 4 new genetically modified lines over the course of the 5 years, this makes approximately 10,000 adult fish. In addition, we have listed a maximum number of 2000 of these fish that might be kept on an alternative breeding protocol, depending on their genetic background.

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

We will carefully design our experiments so that we use appropriate numbers of embryos for each experiment. Where necessary and possible, we will carry out pilot studies to determine the number of embryos required to achieve robust statistical analysis. If we require assistance in our experimental design, we will make use of available guidelines and online tools such as the NC3Rs EDA (https://www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda) and PREPARE guidelines (https://norecopa.no/more-resources/experimental-design-and-reporting/). We will ensure

that our publications conform to the ARRIVE guidelines: https://www.nc3rs.org.uk/arrive-guidelines.

To make our experiments robust, we will control for variability in the following ways:

We will reduce environmental variability by carefully housing breeding adult fish in the dedicated zebrafish facility and by keeping genetic background constant within each genetically modified line of fish.

We will assess normal levels of variability within experiments via pilot experiments, allowing us to select appropriate statistical methods and number of embryos.

We will reduce bias by randomly selecting embryos collected from a pool of breeding adults and, when possible, by assigning treatment and control groups in a way that is unknown to the person analysing the data (blinding).

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

We are using several methods to reduce the numbers of adult animals used. First, we will share relevant fish stocks with other users within the facility. Second, we will try to limit repeated breeding to once per week to optimise breeding performance. Third, we will minimize the generation of embryos wherever possible for our experiments. Fourth, we will freeze sperm from genetically altered lines of zebrafish for longer-term storage.

Refinement

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

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

We use the zebrafish because (1) its anatomy and genetics are a good model for other vertebrate species; (2) its embryos are externally fertilized and can be obtained without harm to the mother; (3) they are large and near transparent, facilitating imaging studies.

We don’t envisage any suffering in licensed animals beyond the mild procedures described above. We will only use zebrafish embryos younger than 5 days old for our experiments, which are not yet capable of independent feeding or complex cognitive behaviours. We will aim to reduce any potential suffering of these embryos by promptly killing them using a humane, approved method at the end of the experiments and, where possible, by anaesthetising embryos that are sufficiently developed to be capable of initiating movement during imaging (those above 18 hours old).

Adult fish will be housed in a dedicated centralised zebrafish facility, where they will be looked after by full time staff, who will ensure their welfare. Numbers of fish per tank, water quality and food quality and quantity will be optimised and carefully controlled.

We have refined our fin clipping to remove only a very small region of the fin, that alleviates the need to provide analgesia.

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

It is essential that we use a vertebrate model for our work, as we want our results to translate to the manipulation of mammalian stem cells in culture. The zebrafish embryo is the most refined vertebrate model possible for the work that we propose. The zebrafish embryo is also an ideal model system for studying organ development, since they are small, transparent, develop rapidly and it is possible to alter their genetics in a reasonably straight-forward way.

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

We don't envisage any suffering in the vast majority of licenced animals beyond the mild procedures described above. Moderate effects might occasionally arise due to the genetic alteration of the fish, as described above. If that occurs, the animals will be promptly killed using a humane method. We will only use zebrafish embryos younger than 5 days old for our experiments, which are not yet capable of independent feeding. We will aim to reduce any potential suffering of these embryos by promptly killing them using a humane, approved method at the end of the experiments and, where possible, by anaesthetising embryos that are sufficiently developed to be capable of initiating movement during imaging (those above 18 hours old).

Adult fish will be housed in a dedicated centralised zebrafish facility, where they will be looked after by full time staff, who will ensure their welfare. Numbers of fish per tank, water quality and food quality and quantity will be carefully controlled.

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

Appropriate experimental design for our experiments in unlicenced embryos under 5 d.p.f. will be carried out, as described in the 'Reduction' section above. Licenced animals older than 5 days old will only be used for establishing genetically altered zebrafish for subsequent breeding. These will be housed in a dedicated centralised zebrafish facility, where they will be looked after by full time trained animal technicians, who will ensure their welfare, in line with their training on best practise. There are several resources to inform us about the current research on refinement of procedures (e.g. https://norecopa.no/species/fish/, https://nc3rs.org.uk/3rs-resources/zebrafish-welfare, https://www.lasa.co.uk/current_publications/). These will be taken into account when deciding on the most appropriate method for procedures..

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

Advances in 3R tools are internally circulated. We can also access advances via the NC3Rs (https://nc3rs.org.uk/resource-hubs) and Norecopa website pages (https://norecopa.no/databases- guidelines). If scientifically appropriate advances in 3Rs arise in the course of the project, we will seek advice from the named veterinary surgeon and named animal care and welfare officer about whether and how to implement them.

31. Mechanisms of organ development and disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

organ, morphogenesis, epithelium, optogenetics, mechanics

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?

To further knowledge about the mechanisms of organ development and how these might relate to 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?

Most organs in the body arise from simple tube-like structures, made from specialised cells called epithelial cells. These cells all point towards the fluid filled cavity (lumen) found at the centre of the tube. The strict organisation of epithelial cells into this rosette-like formation is critical for the function of the organ. If cell organisation is disrupted either during development or later in life, it can lead to disease. Therefore, understanding the fundamental mechanisms driving epithelial tube/cavity development is very important for the appropriate design of bioengineering strategies to grow organs synthetically. It is also important for identifying potential causes of epithelial organ disease so that these might be better targeted therapeutically.

Many factors are important for the normal development of epithelial tubes/cavities. Both chemical signals and mechanical environment can interact to determine the behaviour of cells during organ development. We plan to use a technique called optogenetics, which allows researchers to use light not only to see individual cells and their internal components as they build organs but also to manipulate them. This will allow us to change the signalling within particular cells and test the consequences on the mechanics and development of the whole organ. One of the organs that we will look at is the early brain of the zebrafish, which arises from an epithelial tube called the neural tube. This is a good model for the part of the human neural tube where it is common for birth defects such as spina bifida to occur. We hope that better understanding of the mechanisms behind neural tube formation will provide clues as to how these diseases can be avoided in future.

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

The overall goal of this work is to further the knowledge that our lab has uncovered about the fundamental processes that are necessary for normal organ formation within vertebrate animals and how these might relate to disease. This will provide important knowledge for future bioengineering strategies. In addition, this work should provide clues as to what happens to cells at the onset of diseases such as epithelial cancers and neural tube disorders, which may influence therapeutic strategy in the long term. Our work will also further develop an important bioscience tool - the ability to reversibly manipulate proteins and signalling within single cells within a whole organ using light (optogenetics). This can be used for a wide range of biomedical research.

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

The knowledge gained from our research will enable us and other researchers to better design bioengineering approaches with the long-term aim to grow organs outside the body. The knowledge gained will also benefit researchers in the field of organ disease to better understand how the mechanisms of organ development might link to initiation of disease. In the long-term this might lead to more targeted therapeutic approaches. In the short-term, the further development of technology, especially of optogenetic techniques in vivo (in the living animal) will benefit a diverse range of research groups, who can use this technology to facilitate a wide range of scientific research.

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

We widely share the results of our work via regular presentation of our data at international conferences. We share the techniques used by contributing to international practical workshops. We upload our data on pre-print servers and publish our results in open-access journals. We upload our datasets on open-access sites and share reagents and code used in publications via e.g. online depositories. We also share experimental outputs of publications such as plasmids via open access physical depositories and genetically modified lines of fish via e.g. sperm freezing.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 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.

We will use zebrafish embryos for this research because they are transparent and develop rapidly. The neural tube in the brain is formed over approximately 14 hours. This means that we can put the in-tact embryo under a specialised microscope and can easily image the whole process of vertebrate epithelial tube formation in their brain within the course of one experiment. The early zebrafish brain is also formed via a similar process to lower spinal cord formation in mammals, making it a good model of this process. All of our experiments will be carried out on embryos under 5 days old, which do not require a licence from the Home Office. To generate these embryos, it is necessary to generate and maintain a breeding population of adult fish, which does require a licence.

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

The adult zebrafish will be housed in a dedicated aquarium within the department, run by trained staff. We will generate genetically altered zebrafish by introducing modified genetic material at the 1 cell embryo stage and growing these embryos to adulthood. In order to know which fish contain genetic alterations we sometimes need to carry out genetic analysis via e.g. cutting a small portion of the fish's tail fin under general anaesthetic and analyse the genetic code inside this tissue. The fish is then kept in a separate tank with fresh water and the fin then regrows relatively quickly (within approximately 2 weeks). Where appropriate, other methods of genotyping may be used, such as swabbing the surface layer of the skin. Adult fish will be maintained until a maximum of 30 months of age (although we aim to only keep adult fish until 18 months of age in the majority of cases). During this time, adult fish will be bred in specialised breeding tanks to enable the production of genetically altered zebrafish embryos.
We very occasionally need to anaesthetise fish for the collection of eggs and sperm. At the end of the protocols fish will be humanely killed or supplied to other project licences or recognised establishments with the authority to breed and maintain genetically altered zebrafish of this type.

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

We do not usually expect there to be adverse effects to adults caused by the genetic alterations that we introduce. However, for example in the case of introducing optogenetic genes, it is sometimes possible that, due to basal activity of these genes, some adverse effects might arise in the fish. If this occurs, we would expect such effects to be mild (such as thinner bodies). However, it is possible that sometimes moderate effects might occasionally arise (such as significantly bent body-axis, which might effect swimming). It is also possible that the survival of larvae to adulthood might not be as high in some genetically modified lines when compared to wild type lines. If moderate effects were to occur, we will humanely kill the affected fish. We do not expect there to be any adverse effects from breeding the zebrafish. It is unlikely but possible that fish might develop an infection following removal of a small part of the tail fin, in which case we will humanely kill the fish. For both genotyping and sperm/egg collection, it is possible that fish may not recover from anaesthesia but this is very unusual (less than 1%).

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

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

Zebrafish. Mild: 90%. Moderate: 10%

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 focus of this project is understanding how organs are built and how disease is initiated inside a living animal. It is important to use an animal rather than looking at cells in culture because the physical forces and interactions between cells are very different in a culture system. Therefore, it is

only possible to fully understand the cellular behaviour during organ development by looking inside an in-tact animal. However, we are able to carry out some of our research using cells grown outside an animal (cell culture) – for example we have recently published a paper using cell culture to determine how cell-cell contact is involved in initiating cell polarisation and have accordingly reduced the number of animals used in our research. As we investigate the animal model alongside this culture system, we will learn whether more of our work can be carried out in culture.

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

We have introduced the use of cell culture models in our lab, which we have already used to replace some of our animal experiments. This resulted in the discovery that the centre point of epithelial organs is directed via aligning adhesions between cells with the plane of cell divisions. Carrying out this work in culture reduced our anticipated animal usage in our last licence by roughly 25%.

Why were they not suitable?

It is not possible to entirely replace animal experiments for this project since it is important to test whether the principles identified in cell culture are the same in vivo. This is especially important when considering the mechanical aspects of organ formation, which will be dependent on the physical environment of the organs.

Reduction

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

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

All of our experimental work will be carried out in zebrafish embryos younger than 5 days old (which are not protected under The Animals (Scientific Procedures) Act 1986. Animals older than 5 days old will only be used for establishing genetically altered zebrafish for subsequent breeding. The number of adult animals used is therefore solely related to the numbers required to maintain sufficient breeding stocks of animals. We have listed the maximum number of adult zebrafish that we would use for breeding purposes over 5 years to be 10,000. I calculated this based on requiring approximately 120 fish per new generation of fish for each genetically modified line. We will make a new generation for each line every year (5 generations per line). In addition, when generating new genetically modified lines, the F0 embryos need to be genotypically screened. Therefore, approximately 200 additional fish may be required per new line at the F0 stage to find appropriate founders to generate the F1 generation. Based on raising 15 established lines and generating 4 new genetically modified lines over the course of the 5 years, this makes approximately 10,000 adult fish. In addition, we have listed a maximum number of 2000 of these fish that might be kept on an alternative breeding protocol, depending on their genetic background.

However, we anticipate that the numbers that we will actually use should be significantly lower, since we are committed to actively reducing animal usage (see below).

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

Licenced animals older than 5 days old will only be used for establishing genetically altered zebrafish for subsequent breeding. Therefore, it is not possible to reduce the number of licenced animals used via experimental design. However, we still aim to reduce the number of unlicenced zebrafish embryos under 5 days old being used in our experiments via experimental design. There are several experimental design assistants and guidelines available to help us with appropriate design of each experiment. For example, the NC3Rs EDA (https://www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda) and PREPARE guidelines (https://norecopa.no/more-resources/experimental-design- and-reporting/).

We will also ensure that our publications conform to the ARRIVE guidelines: https://www.nc3rs.org.uk/arrive-guidelines.

For example, to make our experiments robust, we will control for variability in the following ways:

We will reduce environmental variability by carefully housing breeding adult fish in the dedicated zebrafish facility and by keeping genetic background constant within each genetically modified line of fish.

We will assess normal levels of variability within experiments via pilot experiments, allowing us to select appropriate statistical methods and number of embryos.

We will reduce bias by randomly selecting embryos collected from a pool of breeding adults and by assigning treatment and control groups in a way that is unknown to the person analysing the data (blinding).

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 several methods to reduce the numbers of adult animals used. First, we will share relevant fish stocks with other users within the facility. Second, we will try to limit repeated breeding to once per week to optimise breeding performance. Third, we will minimize the generation of transgenic lines and use wild type embryos wherever possible for our experiments. Fourth, we will freeze sperm from genetically altered lines of zebrafish for longer-term storage. We will also carry out efficient genotyping and raise fewer fish per generation wherever possible. Using these methods, we have already successfully reduced our animal usage in our current PPL, so far reporting far lower numbers than the maximum level on our licence.

Refinement

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

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

We will use zebrafish during this project, in order to establish and maintain breeding stocks of fish that we will use to produce the genetically altered embryos.

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

To understand how organs develop within vertebrate organisms it is necessary to use vertebrate animals. The zebrafish embryo is the most refined vertebrate model possible for the work that we propose. The zebrafish embryo is also an ideal model system for studying organ development, since they are small, transparent, develop rapidly and it is possible to alter their genetics in a reasonably straight-forward way. This means that they are an ideal system for using the optogenetic approaches that are integral to this project.

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

We don’t envisage any suffering in the vast majority of licenced animals beyond the mild procedures described above. Moderate effects might occasionally arise due to the genetic alteration of the fish, as described above. If that occurs, the animals will be promptly killed using a humane method. We will only use zebrafish embryos younger than 5 days old for

our experiments, which are not yet capable of independent feeding. We will aim to reduce any potential suffering of these embryos by promptly killing them using a humane, approved method at the end of the experiments and, where possible, by anaesthetising embryos that are sufficiently developed to be capable of initiating movement during imaging (those above 18 hours old).

Adult fish will be housed in a dedicated centralised zebrafish facility, where they will be looked after by full time staff, who will ensure their welfare. Numbers of fish per tank, water quality and food quality and quantity will be carefully controlled.

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

Appropriate experimental design for our experiments in unlicenced embryos under 5 d.p.f. will be carried out, as described in the 'Reduction' section above. Licenced animals older than 5 days old will only be used for establishing genetically altered zebrafish for subsequent breeding. These will be housed in a dedicated centralised zebrafish facility, where they will be looked after by full time trained animal technicians, who will ensure their welfare, in line with their training on best practise. There are several resources to inform us about the current research on refinement of procedures (e.g. https://norecopa.no/species/fish/, https://nc3rs.org.uk/3rs-resources/zebrafish-welfare,

https://www.lasa.co.uk/current_publications/). These will be taken into account when deciding on the most appropriate method for procedures..

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

Advances in 3R tools are internally circulated. We can also access advances via the NC3Rs (https://nc3rs.org.uk/resource-hubs) and Norecopa website pages (https://norecopa.no/databases- guidelines). If scientifically appropriate advances in 3Rs arise in the course of the project, we will seek advice from the named veterinary surgeon and named animal care and welfare officer about whether and how to implement them.

32. Generation and maintenance of genetically altered mice

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Genome engineering, Validation and breeding, Maintenance, 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?

To generate, validate and maintain genetically altered (GA) mouse models relevant to the study of normal and abnormal biological and pathological processes in mammals. To carry out mutant generation, molecular validation breeding as a service to scientific groups throughout the UK and occasionally abroad.

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

Why is it important to undertake this work?

Undertaking this work as part of a standardised genome engineering and breeding programme takes advantage of a large group people with strong technical skills. It will result in the efficient use of animals and is essential to increase reproducibility and maintain the genetic integrity of mice used in genetic research. Importantly, this programme offers the opportunity to reduce numbers for specific projects.

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

2) Quality controlling GA strains of mice ensures that subsequent experimental cohorts bred of these strains have a known, reproducible genetic alteration. Conclusions about the phenotype caused by the introduced genetic alteration are easier to interpret and more robust and reproducible using controlled strains rather than non-standardised or uncontrolled backgrounds. Evolution of molecular biology techniques, enhances the depth of molecular validation and thus reveals potential variability for the quality of new genetic alterations. Moreover, new technologies direct modify embryos rather than cultured embryonic stem cells, which also requires continual improvement in the depth of molecular validation of new alleles to animal work.

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

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

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

1. The main output will be new genetically altered animal models designed after an in-depth assessment of the requirements of the scientific project, the existing published resources, and in line

with the state of the art technologies in the field of animal modelling for biomedical research.

2. A number of mouse lines from peer reviewed initiatives aimed at refining and delivering better mouse models for biomedical research will be generated on this licence. These lines will be generated on this PPL. It is anticipated that around 30 new genes will be modified per year, with the potential for multiple different alleles being produced for each gene. All mouse strains from this initiative will be archived and freely available to the scientific community. The new models will undergo full quality control (QC) of the modified alleles, including understanding what genetic changes have been made and checking for the off-target effects of genome editing. This ensures correct interpretation of how the genetic change impacts the phenotype and ensures lines with unwanted changes are discontinued in a timely manner. Initial breeding of newly generated GA lines will collect welfare data which will be disseminated to the users of these strains.

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

may be bred for further use and dissemination to the biomedical community under different authorisation.

4. A number of mouse lines produced on this PPL will be part of peer reviewed projects but will not be made publicly available.

The scale of genome engineering work in the service also yields a body of knowledge that is broadly applicable to the genome engineering field for improving both efficiency of model generation and validation. The information will be the object of regular method and commentary publications as well as inform the content of courses as well as at diverse relevant academic meetings (i.e. International Society of Transgenic Technologies or International Mammalian Genome Society) and on line.

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.

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

The expected benefits of this project are production and validation of around 150+ high-quality GA mouse lines and tissues thereof for research in biomedical sciences.

In the short term this will lead to more reproducible experimental data. Longer term this will have lasting impacts for basic research, with the availability of thoroughly validated mice as models of aspects of human disease and pre-clinical models for drug development.

Significant numbers of new lines generated through peer reviewed programmes will be available every year to the global biomedical research community.

Deeper understanding of variability of genome editing outcomes which is a valuable knowledge to develop 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. Several members of the teams involved in the genome engineering process in the Centre also contribute to national and international specialist networks and have written advice on genome engineering and model validation processes and regularly answer questions through dedicated mailing lists or receiving ad hoc requests.

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

Several members of the service team, and senior staff in the Centre, speak at conferences on current genome engineering and validation techniques, reproducibility in mouse research, focussing on the optimisation of the design of model 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 processes 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: 186,000

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.

Mice are needed for this project as it is a service licence to generate and validate genetically altered mice to other research programmes. There is no need to age mice on this licence, but we will need mice at all other life stages for breeding and the techniques required for generating new GA lines.

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

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

Mice will be born, they will have an ear clip taken for identification and 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. Any mice not moved on to other projects will be killed. Embryos may also be harvested for tissues after killing.

Mice will 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. 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.

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 direct the genetics changes in these embryos required for the new mouse model, they are put back into an expectant mother mouse by a 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, for which pain relief will be given, embryos are inserted into the reproductive tract. Mice are born 19 days later and when they are old enough, a small ear clip is taken in order to check that they are our expected new mouse model. This procedure is only associated with momentary discomfort.

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).

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 concerns are carefully monitored and if they become clinically apparent and reach human endpoint, the animal is killed. The techniques used in this project have been designed to minimise suffering and animal numbers. The techniques are all well used and the likelihood of adverse effects is known to be low.

Mice used in this project will either be transferred to another project so the scientist who asked for them can begin their studies or they are humanely killed. Many models will be produced under community programmes or under the umbrella of funders that have a policy of systematic sharing of research models. We also encourage scientists to add their mice to public mouse archives so that they are available to the rest of the scientific community and don’t need to be remade elsewhere.

Expected severity categories and the 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 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?

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.

New mouse models are only created if a PPL with prior ethical and or peer reviewed assessment already exist that justifies their use. For community programmes that support the use of mouse models the projects are subjected to a review committee that ensures that alternative non-animal methods (such as induced pluripotent stem cells or organoids) are used to investigate the gene/mutation functions where possible before the mouse model is requested and that there are no alternatives to furthering research other than to generate a mouse model.

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 and we will participate in projects where a better understanding of a gene involved mammalian biology or disease can only be generated through the use of an appropriate model like the mouse.

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.

As such reagents become available we will seek replacements of reagents from animal origin with materials from non-animal origin.

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 will have be done as part of the peer review of projects or on the PPL's that the mice will be moved onto. New mouse lines generated through the funded programmes will need to justify the use mice as part of the application process, and other lines will have been the object of prior ethical or peer review process prior to their generation. Evidence of peer review and the absolute requirement to use animals is sought, reviewed and recorded as part of the commissioning process. 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?

For specific GA models, in many cases non-animal models are used alongside or prior to work in mice. Mice are also used to generate cells for use in ex-vivo tissue culture. Mice

are only used at a point where they are necessary to adequately answer questions that cannot be studied in other, non-animal systems.

Reduction

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

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

1. Genetic modifications are delivered through the microinjection of 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.

Typically, 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 40-80 potential founders. After molecular characterisation 2-4 founders bearing the desired modification, are mated to wild type animals, each of them producing 20-100 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 these different level of difficulty. The efficiency of the protocols are reviewed regularly to allow for the revision of the number of embryos inputted at the onset of new projects. The service accepts projects according to its overall capacity in such a way that it is in continuous use throughout the year.

This equates to 56 females + 50 potential founders + 60 progeny = 166 animals per session if they yield 3 positive founders, 106 if they do not. The service has a realistic capacity of 200 microinjection sessions/year over 5 years; Total number of animals over the course of the licence: 150,000.

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.

2. Genome editing GA line that are in intermediate phases of generation requires between 2-5 sessions of micro-injection, each using approximately 20-50 heterozygous females. This means that between 110 and 260 mice need to be generated per line, an average of 185 mice. Approximately 15 lines will be bred per year for this purpose. 13,875 over 5 years.

3. 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 15 allele combinations will be needed per year for this purpose corresponding to 7,500 animals over the course of 5 years.

Overall, this equates to approximately 180,000 mice over 5 years, approximately 15% 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 needed will be under constant review.

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

The design of each experiment is discussed at the outset with members of the teams that implement the work covered by this licence. The number of microinjection session required are discussed according to the efficiency of the production of positive founders. For example, a point mutation which have very high level of success rate will be the object if a single electroporation session before potential founders are analysed, while two to three microinjections sessions maybe performed for the insertion of a large DNA cassette in a locus so the sufficient potential founders are produced to yield a reasonable chance of producing 2-4 positive founders.

We calculate individual superovulation and breeding strategies taking into account several factors. Including 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.

When more efficient in terms of yield, hyper ovulation and IVF will be used to increase the production of one or two- cell embryos for genetic manipulation when the quality of embryos produced by this method is 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.

All lines generated as part of many projects are freely available to the scientific community so are sometimes shared between several 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.

We also will encourage the depositing of the lines into a public repository as much as possible, with all mice being generated through the community programmes being available through suitable repositories.

We will pilot 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.

Refinement

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

Which animal models and methods 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 is not possible to know what the adverse effects will be. Specific humane endpoints will be put in place based on an extrapolation of the symptoms in humans and predicted adverse effects in mice. For example, where humans have a neurological disorder with movement abnormalities, we might expect tremors or gait abnormalities in mice.

We will prioritise methods where the production of homozygotes or trans-heterozygotes founders are inhibited when targeting genes that are lethal or very deleterious or associated with severe phenotypes when both alleles are modified. 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.

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

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

Projects will be carried out on this licence when it is necessary to assess the gene function in a mammalian system. The ability to modify genomes in a complex way is more advanced in mice than in other species, even if simple modifications are now possible across other species due to the advent of CRISPR/Cas9 technology. Additionally, and critically for genetic studies, there are a large variety of mouse inbred strains in which a

genetic modification can be assessed in the context of a standardised genome. Such inbred strains are significantly rarer or not available for the majority of species. Mice have a wealth of baseline data from which to draw comparisons with, both at a phenotype and genetic level. For bespoke breeding projects not part of large funded programmes, evidence of peer review and the necessity to use mice will be required prior to the generation of new GA lines on this service PPL.

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.

We have introduced a single use policy of needle of 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, 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 has full AAALAC and ISO (9001:2015) accreditation. To conform to these standards we must ensure a high level of quality control on all aspects including husbandry and administrative processes.

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

For terminal procedures routes and volumes for administration of substances are taken from LASA guidelines.

All procedures, including husbandry tasks, are carried out to strict standard operating procedures.

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

The PPL holder and managers working on this licence will attend national and international conferences 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.

33. Cancer progression in skin and breast: from tissue homeostasis to advanced cancers

Project duration

5 years 0 months

Project purpose

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

Key words

cancer, stem cell, microenvironment, therapy

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?

Overall aim

The overall aim of this project is to identify novel strategies for preventing or limiting 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?

In a tumour, cancer cells interact not only with each other, but also with their surroundings, including non-cancerous cells and non-cellular structural components. These interactions

have a crucial impact on tumour growth at primary and secondary sites, can interfere with therapy response, and can mediate the occurrence of drug resistance.

In cancer the homeostasis of a tissue and also the crosstalk between different cell types is disturbed. At advanced stages of the disease, cancer cells escape from the primary tumour site and manage to survive and grow in a secondary organ. Secondary cancers, also called metastatic disease, are responsible for 90% of cancer-related deaths.

Furthermore, cancer patients have a 20% increased risk to develop a second primary malignancies in comparison to the general population. These cancers are independent from the original cancer. The occurrence of second primary cancers are a particular concern of oncologists in patients receiving long-term anticancer therapies as they can be a side effect of the original anticancer treatment.

There is an unmet need to better understand how disturbance of tissue homeostasis and counter- intuitively anticancer therapy can lead to tumour initiation and progression.

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

The outputs of our work will mainly be in form of scientific papers published in peer-reviewed journals. We will also disseminate findings at scientific meetings, and to the wider public through traditional press releases, as well as web-based, social media and public engagement events. Additionally generated genome wide data sets and biological resources such as cell lines and molecular reagents will be made available to the research community.

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

In the short- and medium-term benefits will be in advancing our knowledge on tissue homeostasis and cancer progression. The data acquired in this project will benefit the research community, such as cancer researchers but also those studying tissue regeneration and drug development. The anticipated benefits (and beneficiaries) are:

(a) to provide novel information on the mechanisms of tissue homeostasis, disease progression and metastatic spread, in particular the crosstalk between tumour cells and the microenvironment and how this can influence the response and resistance of tumours to therapy and the development of second primary cancers. Within the time-frame of the new licence (5 years), this information will benefit researchers in the field.

(b) to increase our knowledge of novel targets involved in tumour progression, and in particular to develop strategies for targeting the permissive tumour-microenvironment crosstalk. Within the time- frame of the new licence (5 years) this information will benefit researchers in the field of cancer research and drug discovery. It is likely that promising therapeutic strategies identified in this project can be moved towards a drug discovery and translational development stage within 5 - 10 years.

(c) the development and improvement of in vivo protocols and models to interrogate the interaction between tumour cells and the microenvironment. Within the timeframe of the new licence (5 years) this will benefit scientists studying the events.

(d) an improved understanding of how stromal cells and extracellular matrix components contribute to tumour progression will enable the improvement of 3D in vitro models. Publishing such protocols will, within the time-frame of this licence (5 years), contribute to reducing the number of animals used in research, as it will allow for example a better pre-testing of potential anticancer agents in vitro.

In the long term, our findings will benefit patient outcome.

First, by better understanding how therapies induce second primary cancers and whether they are preventable and second, understanding whether targeting specific non-cancerous but tumour- promoting cells, is a therapeutic option in addition to targeting cancer cells by themselves.

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

Our findings will be made available to other scientists through peer-reviewed publications, presentations at internal, regional, national and international conferences, as well as at smaller meetings with collaborators.

Species and numbers of animals expected to be used

- Mice: We expect to use approximately 1730 mice over 5 years.

Predicted harms

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

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

In this project we will use juvenile and adult mice. Mice are the most common model organism for preclinical studies, their genome is similar to the human genome (99%), a good genetic/molecular toolbox is available. Using mice allows

- the study of disease mechanisms in a complex organism (including the response and action of the immune system),
- the understanding of the mechanistic causes and pathways underlying human disease
- permits the development of efficient and targeted treatments. Mouse models have been
- successfully used to validate drug targets and to determine efficacious and safe dosage schemes

  for treatments.

Our studies involve analyses of complex interactions between tumour cells and non-cancerous cells, such as immune cells and fibroblasts during cancer initiation and progression in the body.

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

Protocols can be divided into A) tissue homeostasis in the skin and B) tumour progression with a focus on skin and breast cancer.

To study skin tissue homeostasis we graft skin cells with certain characteristics into the mouse skin and can monitor how these cells generate a new skin (takes on average a month) and how the old or new skin respond to external challenges. Challenges could be for example the introduction of a wound to monitor skin regeneration or the exposure to UV light.

Tumour studies will include the injection of cancer cells or treatment of the skin with tumour-inducing reagents or UV light. Most studies will last between 10 and 40 days in case of murine cancer models, but may take longer in case of human cancer models, which often have a longer time-span before tumours start growing. Prior or post tumour cell injection animal may be treated with test agents (e.g. anticancer therapeutics) that promote or inhibit tumour growth to study the effect of therapies on tumour growth and also non-cancerous cells. In some cases tumour cells may be injected intravenous to give rise to tumours in the lung, or intraperitoneal for growth in the abdomen to mimic growth in secondary organs. During the studies we may also take blood samples.

Studies will be terminated after 180 days maximum, however, most studies will be of shorter duration. They will be designed to use a minimum number of mice that would produce satisfactory results.

At the end of an experiment, mice will be humanely killed and samples such as tumours, skin, lungs, lymph nodes and blood will be taken for processing and detailed analysis. Besides analysing tissue sections, we can also isolate cells and culture them in vitro to conduct further studies.

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

Mice will develop tumours over time, depending on the tumour type and injection site this can be between ~14-90 days, however, the majority of animals are not expected to show signs of adverse effects that impact on their general well-being. Animals that develop tumours will be closely monitored for signs of pain and distress such as loss of appetite, and abnormal behaviour and gait. General health and maximum allowed tumour sizes will be regulated by strict guidelines. A scoring system is used to help monitoring general health and tumour growth and gives guidance when to terminate the experiment if necessary, e.g. mice may have a loss of weight of up to 15% for 72 hours.

In cases a wound was introduced into the skin, the skin will begin to heal within 12-24 hours. Infection is unlikely since sterile punches and needles are used, however, we do have a scoring system for lesions and infections in place if necessary.

Some mice will be subjected to small surgeries in the skin (e.g. skin wounding, implantation of small tumour pieces, fixation of a cover to allow secure wound healing). Surgery will induce transient moderate pain. However, sterile techniques, analgesia and anaesthesia will be used to minimize stress, pain and suffering.

Mice may be treated with test agents (e.g. anticancer therapeutics). We anticipate only minimal toxicity of these substances, as they are either of low toxicity or will be administrated at therapeutic levels.

Despite not anticipated we will look out for signs of toxicity (loss of body weight, lethargy) and the development of abdominal bruising, irritation or tissue damage at the site of injection as result of re- current substance administration and might interrupt or terminate dosing if necessary.

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

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

The majority of protocols is classified as moderate as they may include small surgical procedures under anaesthesia in the skin, which can induce transient moderate pain (~40%)

Mice developing lung or intraperitoneal tumours will always be classed as moderate (~20%).

All other mice are expected to have mild adverse effects, e.g. due to the development of a subcutaneous and mammary fat pad tumour (~40%).

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

- Killed

Replacement

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

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

Cancer research has made enormous progress by exploiting tumour cell lines that can be manipulated in vitro. However, during metastasis the complex interactions between different cell types, particularly the immune system, and non-cellular structures cannot be recapitulated in vitro. Similar restrictions can be found when studying the homeostasis of the skin, which involves a crosstalk between tissue- resident stem cells and other skin cell types (e.g. keratinocytes, melanocytes, fibroblasts).

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

Despite using in vivo models, we aim to develop better in vitro models to improve for example the selection of therapeutics and identification of novel targets. To the end we study and improve co-culture systems including not only cancer cells but also normal cells and non-cellular structures. Furthermore, we use these cells in so called organoid cultures which better recapitulate the 3D environment of the skin or a breast tumour. These improved models help us to replace animals wherever possible. In addition to in vitro

models, we use in silico methods (e.g. interrogation of human cancer databases and clinical data sets).

Why were they not suitable?

There are numerous stages during cancer progression, which are difficult to replicate in vitro due to the involvement of different cell types, different structures, and physical cues. This includes generation of blood vessels, the escape of tumour cells from a primary tumour to a secondary organ (metastasis) and the complex interactions between the tumour cells, normal cells, and non-cellular structural components. Furthermore, we are particularly interested how non-cancerous cells behave and evolve during cancer progression. These cells are often difficult to culture in vitro without inducing a change in their characteristics they would have in the original tissue.

Reduction

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

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

The estimation of the total number of mice is based on our experience from previous research combined with the use of statistical tools to calculate optimal and minimum number of animals for each experiment.

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

The following steps are taken :

(a) in-house statistical experts (e.g. the Mathematics and Statistics Help (MASH)), collaborators and NC3R's Experimental Design Assistant to ensure that we are using optimal and minimum group sizes,

(b) optimised procedures are used to reduce the number of mice and to reduce experimental variability.
(c) ensure that each experiment is maximally analysed.

(d) plan and coordinate experiments to harvest and collect as many organs for analysis as possible to fully analyse samples from each experiment.

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 (a) perform pilot experiments to determine humane endpoints and optimise establishment of models, and (b) will mostly use wildtype mouse strains instead of genetically altered strains that require more complex breeding.

Refinement

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

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

We will only use inbred mice in this project. In this project we will use mice with either an intact immune system or a reduced immune system. The latter will allow us to study human cancer cells in mice. The growth of tumours is a routine and refined procedure in cancer research, resulting in reproducible tumours. We will minimise animal suffering by robust and regular checks for animal well-being and tumour growth and sizes. We will make every effort to keep the tumour models at subclinical levels.

Some mice will be subjected to surgery, but analgesia and anaesthesia will be used to minimize stress and suffering. Therapeutic drugs will be used accordingly to safe toxicities and therefore we expect high tolerability of the regimens. If animals show signs of mild adverse effects animals will be managed to ameliorate symptoms and, if necessary, will be humanely killed. If necessary we may perform pilot experiments to determine humane endpoints and thereby reduce the adverse effects that an animal may suffer.

Furthermore, only experienced staff will perform optimised procedures to minimise the severity and suffering of animals.

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

Mice are the least sentient species that are appropriate for this work, particularly in drug development studies and for studies based on the response and action of the immune system. Our studies involve analyses of complex interactions between immune cells, other stromal cells and cancer cells.
Whenever possible we use subcutaneous injections of tumour cells however, the tumour microenvironment is best replicated in orthotopic sites (skin, mammary gland) and relevant metastatic sites (e.g. lung) as tissue resident cells, even if they are in principle the same cell type, differ between organs.

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

A range of steps will be taken to minimise the severity of procedures. Suffering will be minimised by having experienced and trained staff undertaking the procedures that have been developed over many years and are widely recognised and published as being suitably refined to minimise the level of distress and suffering to the animals. In protocols using surgery, aseptic techniques will be used and pain will be managed by using analgesia. Animal health, adverse effects of procedures, tumour growth and wound healing will be closely monitored using a scoring system with clear follow-on instructions

which can include for example increased monitoring or providing specific supportive measures such as specific food. Tumour sizes are limited to a maximum size and tumour associated-symptoms will be monitored.

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

Throughout this project, we will undertake all animal work in accordance with the principles set out in the Guideline for the Welfare and Use of Animals in Cancer Research. We will also follow best practice guidance from the NC3Rs and the guiding principles for undertaking aseptic surgery from LASA .

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

- I am attending regular meetings of our BSU.
- I receive and follow the newsletter and publications of the NC3R
- I take part in conferences to stay informed about advances in the field of the 3Rs and will implement as much as possible.

34. Neuroprotection and treatment of dyskinesia and cognitive decline in animal models of Parkinson's disease (PD)

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

Parkinson's disease (PD), Neurodegeneration, Neuroinflammation, Neuroprotection, Cognition

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 this project is to use mouse and rat models of Parkinson's disease (PD) to test drugs that have the potential to protect neurons and reduce symptoms associated with the disease, including dyskinesias and cognitive decline.

The aim of this project is to: a) study neuroprotection in PD models by examining various strategies that target the neurodegenerative cascade, b) better understanding of the mechanism behind levodopa induced dyskinesia and focus on its treatment, and c) to understand the extent of the attention deficit and cognitive decline in the rat model of PD, and investigate potential treatments.

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

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

Why is it important to undertake this work?

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease after Alzheimer's. There are no cures but the effects of drugs such levodopa (Sinemet or co-careldopa and Madopar or co-beneldopa) that improve motor symptoms in people with PD are limited to a brief time-frame; after a few years these drugs lose their effectiveness and abnormal involuntary movements known dyskinesia set in. Moreover, there are several non-motor symptoms e.g cognitive decline for which there is cure or remedy. Preventing disease progression or ideally reversing the course of the disease is one the important priorities in the treatment of PD. However, successful treatments for reversing or stopping the disease progression are currently not available. In order to find agents with this capability, it is very important to better understand the pathophysiology of the disease. While, the process of cell death appears to involve oxidative stress, mitochondrial dysfunction and glutamate/nitric oxide toxicity, numerous findings have suggested that it may be linked to alterations in the ubiquitin-proteasome pathway, and/or to inflammatory processes. These processes may act independently of each other or may be involved as part of an integrated cascade such that reducing one or more of these mechanisms may not reduce the disease pathology. Current findings suggest that new avenues for treatment that result in slowing or halting of disease progression may require drug interventions at different stages of the disease pathologies. For example, this might entail treatments to prevent inflammation, oxidative stress and proteasomal dysfunction. It is therefore extremely important to investigate the interactions between these mechanisms to find a strategy to prevent or slow down the neurodegeneration and the progression of PD.

Although dyskinetic movements can be controlled to some extent by careful use of drugs, the dyskinesia brought about by dopamine replacement therapy to treat PD is irreversible and is precipitated with all further doses of dopaminergic drugs. It is therefore very important to focus efforts on strategies that would prevent the priming process for dyskinesia or reduce its expression when it occurs. This work is therefore extremely important because it will examine novel treatments for the treatment of dyskinesia.

Dopamine is also an important neurotransmitter for cognitive function and loss of dopamine is associated with cognitive decline. Few groups have investigated cognitive decline due to dopamine loss and its remediation in rodents. It is therefore extremely important to model cognitive decline in PD, to understand and treat attention and cognitive deficits.

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

The intended output of the project is to produce data to identify novel compounds or compounds with similar pharmacological profiles for use in preventing or limiting PD. The outputs from this work will mainly be scientific papers in peer-reviewed journals, presentations at national and international conferences, meetings with collaborators.

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

Within the timeframe of this licence (5 years), the outputs of the work will benefit other scientists working in this research field. The advances in our knowledge will aid in the development of novel therapeutic strategies for treatment. By increasing our knowledge

and understanding of the mechanisms involved in neurodegeneration, and better understanding of the pharmacology of non- motor symptoms and levodopa induced dyskinesia it might be possible to identify better molecular targets for intervention. Treatment of motor and non-motor symptoms as well as abnormal involuntary movements might render PD a more benign malady. The outputs of this work will also enable us, and others in the field, to develop better in vitro models and, leading to a reduction in the number of animals used in subsequent in vivo research.

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

These will be in the form of seminars both internally and externally within UK and to the wider international audience, and publications. We will also collaborate with other scientific groups in this field to exchange the knowledge obtained using our studies. Biobanking of tissues, blood and plasma would benefit other collaborators and foster further collaborations.

Species and numbers of animals expected to be used

- Mice: 500
- Rats: 1500

Predicted harms

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

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

Adult rodents used in these studies are the most appropriate models of studying Parkinson’s Disease. Rodents have been used extensively in PD research and the models involving neurodegeneration of dopamine neurons triggered by neurotoxins are well validated. Investigations using in vivo models are necessary to understand the complex interactions between mechanisms involved in the neurodegeneration of neurons in the adult brain, and to observe the impact of treatments on behaviour.

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

Animals will undergo behavioural testing and will undergo various treatments that include administration of toxins by injection peripherally or directly into the brain during brain surgery. Animals will be subject to implantation of small pumps which will administer drugs continuously without the need for repeated injections or administration of drugs by oral gavage, intraperitoneal (injection into the gut cavity) or subcutaneous (injections under the skin) routes. In the case of the former, anaesthesia followed by analgesia will be used for the implantation of small pumps, whereas administration of drugs by the latter routes will result only in moderate discomfort.

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

The animals may experience some symptoms of the disease such as un-coordinated movement, circling movements, excessive urination, constipation, weight loss or excessive involuntary movements. Anaesthesia, analgesia will be used to mitigate the pain

associated with surgery and analgesia will be given to reduce the possible post-surgical pain and discomfort. There are also limits to the number and frequency of any injections, blood sampling and behavioural assessment that any one animal can experience. Overall, the severity of this license is expected to be moderate. At the end of the experiments the animals will be humanely killed, and tissues may be used for biochemical investigations.

Expected severity categories and the 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 75% of the rodents used in this project are expected to have effects of a moderate severity as they will experience (a) surgeries to establish models of PD and (b) therapeutic intervention to alleviate symptoms of PD or to modify pathology.

The remaining rodents will experience mild severity as they will experience (a) cognitive training, (b) behavioural assessments, and (c) non-brain administration of neurotoxins vehicles.

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

- Killed

Replacement

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

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

Brain function is extremely complex and neurodegenerative diseases such as Parkinson's disease (PD) present an equally complex neuropathology with associated motor and non-motor symptoms. It is therefore vital to confirm the positive effects that may be apparent in an in vitro situation in a whole organism that display good face, construct, and predictive validity with respect to brain pathology and behavioural outcomes. We will continue to use rodent models of PD as these are well validated and predictive of the efficacy of therapeutic treatment. These studies provide a vital link in the progression of treatments from the preclinical to the clinical environment.

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

We continue to perform studies using cell culture models of PD to investigate the effects of drugs and toxins in both specific neuronal cell lines derived from human neuroblastoma cells and mesencephalic cultures derived from rat. We use in vitro studies to guide in vivo experiments. Similarly, we are continuing our ex vivo analysis of drug activity using enzyme and binding assays and only those agents that have shown neuroprotective efficacy in vitro will be carried forward to in vivo studies. All drugs to be tested in vivo have been tested in silico and in vitro.

Why were they not suitable?

Searches on www.frame.org.uk confirm that there are no alternatives to the use of animals for the investigation of these complex disorders of the brain.

Reduction

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

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

For ethical, scientific rigour and economic reasons, it is important to design animal experiments well, to analyse the data correctly, and to use the minimum number of animals necessary to achieve the scientific objectives, but not so few as to miss biologically important effects or require unnecessary repetition of experiments. The main purpose is to estimate sample size for an experiment or power or effect size if sample size is fixed Type I errors (alpha) or false positive results (type II errors, beta) arise from sampling variation. A power analysis helps us to estimate the correct number of replicates to reduce the type II error. For the studies to be carried out under this licence the appropriate group size will be determined by power analysis derived from published papers and using pilot studies to allow robust and statistically significant results.

Except for pilot studies, the in vivo experiments would be designed using a cross-over (Latin-square) and randomisation (animals randomly assigned to different treatment groups) design to reduce the source of variability but at the same time reduce the number of animals. All experiments will be conducted so that they conform to the ARRIVE guidelines (Kilkenny et al., 2010) for subsequent publication.

The following sources will be referred to: NC3rs experimental design assistant: https://www.nc3rs.org.uk/experimental-design-assistant-eda ‘Beware of power failure’: Button et al. (2013) Nat Neurosci, 14, 365-376.

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

Where relevant, factorial experimental designs may be used, rather than the one-thing-at-a-time approach, to maximise the information obtained from the minimum resource. For most of the quantitative experiments, sample sizes may be set using power analysis, generally using a significance level of 5%, a power of 80%, and a least practicable difference between groups of 25%. Otherwise, we may use previous experience (ours, or from the literature) to select sample sizes. In terms of the numbers of animals required, we expect that 6-8 animals per treatment group should be sufficient to obtain the required results. It is important to include sham animals to avoid or reduce observational bias, in randomisation and blinding. Furthermore, as part of good laboratory practice, we may write a protocol for each experiment including: a statement of the objective(s); a description of the experiment, covering such matters as the experimental treatments, the size of the experiment (number of groups, number of animals/group), and the experimental material; and an outline of the method of analysis of the results (which may include a sketch of the analysis of variance, an indication of the tabular form in which the results will be shown, and some account of the tests of significance to be made and the treatment differences

that are to be estimated). We may make appropriate arrangements to randomly assign animals to experimental groups and blind studies. Experiments may be planned so they can be published in accordance with the NC3Rs' ARRIVE guidelines.

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

We will optimise the number of animals used in these studies by (a) keeping abreast of published scientific knowledge, and (b) performing pilot studies.

Refinement

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

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

The neuropathology induced by the toxins we will use reflects that seen in PD and dyskinesia and therefore provides a basis for studying complex biochemical and behavioural changes. With respect to PD, the animal model that best recapitulates human PD is the MPTP-treated primate However, it is unacceptable to use this model at early preclinical stages of therapy. Hence, rodent preclinical models are accepted as most suitable for the initial investigation of symptomatic and neuroprotective treatments.

Rodents are generally considered the lowest vertebrate mammalian species suitable to perform studies investigating symptomatic agents and neuroprotective agents in PD research.

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

Since brain function is extremely complex and neurodegenerative diseases such as PD present an equally complex neuropathology with associated motor and non-motor symptoms, it is impossible to consider alternatives such as yeast cells, organ cultures, brain on a chip, fly models (drosophila) or fish (zebra fish) models. It is therefore vital to confirm the positive effects that may be apparent in an in vivo situation in a whole organism that displays good face, construct and predictive validity. We will continue to use rodent models of PD as these are well validated and predictive of the efficacy of therapeutic treatment. These studies provide a vital link in the progression of treatments from the preclinical to the clinical environment.

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

When surgical procedures are carried out, these will be done according to the best practice using aseptic techniques (use of sterile drapes, gloves and instruments would be mandatory).

To minimise the risk of infection surgery will be carried out to HO Minimum Standards for Aseptic Surgery and/or LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery http://www.lasa.co.uk/pdf/lasa_guiding_principles_aseptic_surgery_2010.2.pdf).

Anaesthesia would be carried out mainly using a gaseous mixture of isoflurane / oxygen to enable rapid anaesthesia and rapid recovery. Animals would be kept under anaesthesia no longer than necessary to complete wound closer and administration sedative and fluid (saline + glucose) – to help rapid recovery and preventing dehydration.

Animals will be housed in heated isolation boxes on fresh bedding covered until animals are fully conscious before they are returned to home cages that contained mashed food / hydrogel nutrient and fresh bedding material.

To monitor pain and discomfort, mouse and rat grimace scale will be used. Peri- and post-operative analgesia will be provided where it is necessary to alleviate pain.

The behaviour tests used are passive, non-invasive and are structured around the normal repertoire of movement for the species. Our group has considerable experience with the techniques and welfare and husbandry required to produce these rodent models and in the design and execution of the behavioural experiments. These are factors that are important in minimising the stress to the animals, maximising their welfare and ensuring that reliable and robust results are produced.

All injection volumes will not exceed those considered in standard published LASA guidelines.

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

LASA guidelines on surgery, injection volumes and Grimace scale from nc3rs will be followed.

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

We will continue to be up to date with www.frame.org.uk and https://www.nc3rs.org.uk.

We regularly attend BSU user forum at our establishment and implement any changes effectively to our studies performed.

35. Novel sensors for detecting changes in lower bowel health

Project duration

5 years 0 months

Project purpose

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

Key words

Sensing probe, Anorectum, Ageing, Chronic consipation, Faecal incontinence

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of the project is to understand the causes of lower bowel dysfunction in ageing and disease. This will be achieved through monitoring the chemical and mechanical fingerprint of the terminal bowel and correlating these findings to alterations in faecal output.

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

Why is it important to undertake this work?

Ageing and age-related diseases are major contributors to dysfunction in the gastrointestinal tract increasing the incidence of chronic constipation and faecal

incontinence. Faecal incontinence affects 1 in 5 people aged 65 years or older and chronic constipation can affect up to 50% of institutionalised older people. Both conditions cause a marked decrease in the quality of life of older people and can lead to social isolation and its associated effects on health. For some, faecal incontinence is a major cause of institutionalisation. At present there are no diagnostic approaches which can predict future bowel dysfunction with strategies focused primarily on the management of the existing condition. This study aims to develop a sensor that can monitor lower bowel function in vivo, and has the potential to be able to predict those at risk of developing a bowel condition, providing the opportunity for earlier interventions to delay/prevent the development of this condition.

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

The development of our novel probe will provide new information about the signalling molecules released from the terminal bowel, their role in the function of the bowel and how these are altered across the lifespan. We strongly believe that the changes in signalling that we plan to measure contribute to the onset of a range of bowel disorders. It is hoped that an analysis of how these molecules change with age and disease in mice and the corresponding changes in bowel function will provide the means to develop a novel screening tool that is capable of predicting deleterious changes in bowel function into the future. This work is a vital step in allowing us to develop the probe towards use in humans. The work we generate will be published in peer-reviewed journals and disseminated in workshops to healthcare professionals and patient groups.

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

In the short-term, the outputs from our work will be beneficial to researchers working in the field of gastrointestinal physiology as it will provide novel insights into how changes in mucosal signalling molecules link to dysfunction in the lower bowel across the life course. The work will also be disseminated to clinicians to help educate them about the drivers of changes in lower bowel function. Local patient groups will also be informed of our findings, as part of our longer-term goal to translate these findings into an early diagnostic test to identify those predicted to develop chronic constipation/faecal incontinence. The dissemination of the findings of our study at scientific conferences will hopefully stimulate others to become involved in research exploring lower bowel dysfunction. In the longer term, the success of this project and the requirement to develop a human sensor will also provide economic benefit to industry partners involved with the development and manufacture of these devices and longer term benefits to patients/the ageing population.

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

We will look to share data generated from our new findings through a publicly accessible repository and through conference presentations. These will provide insight into our successful and unsuccessful approaches whilst conducting the proposed study. The outputs from the work will be disseminated to a variety of different groups, including academics involved in lower bowel health, healthcare technology research, clinicians focused on supporting the treatment of those with bowel disorders and patient groups.

A commitment made as part of the grant application is to translate our findings into humans to provide an early detection device for patients at risk of developing chronic constipation/faecal incontinence. To this end, we have secured collaboration with NIHR Surgical Medical Technology Cooperative to support the clinical assessment of a human

sensor should the results from this study prove successful. Therefore, we anticipate that results from this study will be a key stepping stone on a progression to the development of a novel human screening device for lower bowel disorders.

Species and numbers of animals expected to be used

- Mice: 100 animals of each sex will be used for this study.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 proposal plans to utilize both male and female mice. The mouse has several advantages that make it suitable for studies of lower bowel dysfunction. First, their relatively short lifespan (approx. 2 years) ensures that studies across the life course of the animal can be conducted successfully during a typical cycle of funding (3 years). Second, the signalling molecules used to drive motility in the lower bowel of the mouse are equivalent to those seen in humans, although it should be noted that some of the receptors involved in regulating motility may vary between mice and humans. Third, there is a wealth of literature on the regulation of the lower bowel in young mice on which we can build our life course studies. Fourth, the use of the mouse provides for the future potential to utilize technology to manipulate the mouse genome to further explore lower bowel dysfunction.

Critical time windows exist for the onset of bowel disorders in wild type and transgenic mouse strains. In wild type mice this is typically around 18 months, equivalent to a human aged 60 years old.

However, in transgenic mouse models of age-related disease this critical window may occur at an earlier stage of adult life. Our work, will therefore, focus on both adult and aged mice.

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

Animals will initially be acclimatised to a restraint tube. Following acclimatisation animals will be restrained in tubes and a small probe (diameter of a normal faecal pellet) inserted into the anorectum (last 1 cm of the terminal bowel) to record the chemical and mechanical fingerprint of tissue. This procedure will last for a maximum of 3 minutes. Animals will then be returned to their home cage and faecal output recorded 1 week later. This procedure will be repeated a total of 4 times to span the critical window that is specific to the mouse strain. This chemical and mechanical fingerprint will then be correlated to alterations in faecal output recorded a week later in the same mice.

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

Animals may experience some initial stress at being restrained as part of the acclimatisation process. During the actual procedure animals may experience some minor discomfort in the anorectum following insertion of the sensing probe. This probe will be

inserted for a maximum of 3 minutes. This process will be repeated four times on a single animal with a maximum frequency of once every month, dependent on the strain of mouse.

Expected severity categories and the 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 entire study will be moderate with 100% of mice experiencing this severity. Even though each step of the study is likely to cause only a mild transient discomfort, the severity rating for the procedure has been set at moderate due to the invasiveness of the procedure and the requirement to repeat the procedure four times on any given animal to provide a timeline of changes across the critical window of dysfunction.. This expected severity is based on Annex VIII Animals Directive 2010/63 EU.

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 anatomical structure of the lower bowel is complex consisting of multiple layers of tissues (e.g., mucosa, submucosal plexus, circular muscle, myenteric plexus, and longitudinal muscle). Although isolated sections of the bowel can generate their own motility and are able to secrete and absorb ions and water, their function is regulated by the microbiota that is present in the lower bowel and from central inputs from the autonomic nervous system. Further complexity comes for the observation that our project is looking for changes that occur in lower bowel signalling across the life course of the animal, something that is currently not possible to replicate in the culture dish. Together, appropriate data can only be obtained using intact animals.

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

1) Caco-2 cells (human epithelial cell line).

2) Lower bowel human organoids

3) Ex vivo human intact sections of the lower bowel

Why were they not suitable?

Caco-2 cells were not suitable for two reasons i) they are an immortalised cell line, and therefore, we would not be able to carry out an age comparison; ii) given our sensor is also designed to detect muscular contractions of the lower bowel, an important part of the

project, the lack of muscles means this model is not viable iii) the lack of the central nervous regulation also prohibited its use.

Lower bowel organoids: These provide a more complex model that would dramatically reduce animal usage, but they too lack input from the CNS, structurally are not a precise mimic of the lower bowel and while we have generated organoids from young mice, our attempts in older mice have so far been unsuccessful. For these reasons, this model has been excluded.

Ex vivo human tissue: To replace the use of animal tissue we contemplated utilising the sensor in ex vivo intact sections of the human lower bowel. However, this model was rejected for two main reasons.

i) a main aim of our work is to correlate changes in mucosal signalling with changes in faecal output across the life course and thus would not be ethical to obtain full thickness healthy tissue from human participants and additionally ii) isolated sections of the lower bowel also lack the central regulation, a critical component of the functioning of the bowel.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 is based on previous studies we have conducted, specifically those that used ex vivo monitoring to explore the changes in colonic muscle function and serotonin signalling.

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

Animals will be acclimatised to the restraint device used to hold the mice during their probe procedure. Stress can impact on signalling and motility in the lower bowel, so this procedure is vital in ensuring the data we collect is fit for purpose and to reduce the variance between the different animals. The probe/sensor has been designed to be the same size as a normal faecal pellet and is therefore minimally invasive. In addition, we have trialled the use of the probe in ex vivo tissue using a standard lubricant (KY jelly) to ensure that the use of lubricant to minimise discomfort does not impact on data collection. The design of the sensor and the technology that allows us to record the release of chemicals and changes in motility rapidly and therefore the procedure time can be kept to ≤ 3 minutes. By doing this we feel able to carry our repeated measures across the life course of a single mouse.

These data will minimise the use of animals, increase the power of our results by allowing a direct correlation between signalling/motility changes in the lower bowel and measures of faecal output 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?

Our mice will be culled after their final measurement to allow us to perform protein/RNA analysis and histology on the anorectum and to collect and freeze at -80 C a range of organs and tissues to enable other researchers to utilise them for their 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.

Our recordings will be carried out on both male and female mice, a model we have successfully used previously to study age-related changes in the lower bowel and one that is reflective of changes in the human bowel. After a lot of discussion with our NVS and the Royal Veterinary College, we have decided that the best approach is to carry out the procedure in conscious but restrained mice.

Restraint can be a stressful procedure for mice, but we aim to minimise stress by acclimatising the animals to the restraint tubes prior to experimentation and to keep the restraint as short as possible (≤ 5 minutes). In addition, the sensor will be designed to be the same size as a normal faecal pellet and will be inserted using plenty of lubricant (KY jelly). Although this is an invasive procedure, we believe that it will only cause transient discomfort to the majority of animals. To minimise discomfort we will practice our probing procedure on Sim mouse and also on mouse cadavers before using live animals.

Our main alternative is to carry out the procedure in fully anaesthetised animals. However, the use of a general anaesthetic will impact on the regulation of the signalling molecules release and the control of motility and therefore will be detrimental to the data that we collect. The whole procedure will take longer, and the length of the procedure may impact on our ability to return the mouse to its home cage. Given that social isolation is in itself is a stressor and one that we have previously shown to impact on gastrointestinal tract motility and faecal output, this alternative would lead to the use of many more animals in the study. Finally, we wanted to ensure that the data we obtain stands the best chance of translating into a device for monitoring human lower bowel function and therefore, the use of a general anaesthetic is prohibitive.

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

We contemplated the use of terminally anaesthetised mice but rejected this option for several reasons;

i) By its nature the anaesthetic will change the firing patterns of nerve cells that regulate the secretion of chemicals and the contraction/relaxation of lower bowel muscles and therefore, this approach will compromise the data obtained and make it less likely to be

transferable into humans; ii) A main aim of our study was to follow a mouse across its life course and to correlate changes in signalling with alterations in faecal output. This approach would be prohibitive in terminally anaesthetised mice and would seriously compromise the translatability of the data into humans; iii) Given that we have plans to translate the probe we are testing into human trials we would like the procedure that the animals are subjected to be as close to that of our future human volunteers and so anaesthesia was not a viable option.

The use of animals at a more immature stage of life is not appropriate for a study looking at lower bowel health across the life course. The use of a species that is less sentient would also compromise translatability and ignores our previous studies in the mouse which demonstrated age-related changes in mucosal signalling.

Little is known about lower bowel function in less sentient animals, compared to the mouse model that we plan to use. Mouse GI tract anatomy, signalling molecules and lower bowel function are very similar to that in humans and therefore, this provides the most appropriate model to use. Characterisation of a less sentient animal model would require the need to carry out far more preliminary work to characterise the signalling molecules present in the lower bowel, their regulation of motility and how these processes change with age, with no guarantee that the model would be a suitable proxy for human lower bowel function.

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

Animals will be acclimatised to the restraint device so this will help minimise both psychological and any physical stress. After the procedure, all mice will be monitored for a period of 24 hours for signs of pain or distress (behavioural changes, solitary behaviour, piloerection etc). Any animals showing signs of pain or distress during this period or after 24 hours will be killed humanely using a standard Schedule 1 method in consultation with the NACWO and following the guidance of the NVS if required.

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

In order to ensure best practice, we will follow the essential 10 ARRIVE practice guidelines.

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

We receive regular updates of the latest NC3Rs news through our NIO and will implement any necessary changes as required. I also personally receive updates from NC3Rs via email through a personal subscription.

36. Understanding serosal repair and internal scarring

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

scarring, therapy, abdomen, inflammation, repair

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 discover new medical treatments to prevent the formation of internal scars, or 'adhesions'. We will work out how cells inside the abdomen form these internal scars after an operation and/or infection and then use this knowledge to block the process.

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

Why is it important to undertake this work?

Adhesions are bands of scar tissue that join organs to each other and/or the inner body wall and are a huge problem with extensive health care costs globally. It is proposed that adhesions form in one out of every ten people who have open abdominal or pelvic surgery and incidence of small bowel obstruction due to adhesions post-surgery is 2%. The Surgical and Clinical Adhesions Research (SCAR) group found that 3·5% of patients in

Scotland who underwent open abdominal or pelvic surgery were readmitted within 5 years of surgery for disorders directly related to adhesions, 17·6% for disorders possibly related to adhesions, and 13·1% for operations potentially complicated by adhesions. The cumulative, year-on-year, direct costs of adhesion-related re-admissions due to lower abdominal surgery in the UK are estimated to be over £787 million over a 10-year period. One US study reporting adhesion-related complications resulted in over 300,000 hospitalizations and almost 850,000 in-patient day admissions with a total cost of $1.3 billion (over £1 billion) in one year. Furthermore, in Finland, the annual direct hospital cost for postoperative adhesion-related intestinal obstruction was estimated as over £2million. Surgery, infection and ongoing inflammation are the main drivers of internal scar formation.

Adhesions can result in severe abdominal pain and life-threatening bowel obstruction, as well as causing infertility in women. Unfortunately, there is no treatment once formed apart from further surgery, and present approaches to prevent their formation, such as degradable films and gel barriers used at the time of initial surgery, show limited benefit. Hence, there is an urgent need to develop better therapeutic ways to prevent internal scarring, thus minimising the considerable harm and financial burden associated with this condition.

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

This project will provide new ideas for better preventative therapies for internal scars and work out the best to deliver these therapies into the abdomen. Our findings will be published in appropriate scientific journals, discussed at surgical and basic science conferences and presented at science festivals so that new information about adhesions and their prevention is widely circulated to clinicians, scientists,

industry and importantly the public. In addition, we will share tissue samples, relevant data and reagents with others to progress new discoveries in the field of adhesions.

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

In the short-term, we will provide important information about how the abdomen responds to injury and why adhesions form which other scientists can use to modify and extend their own studies.

Researchers with an interest in injury and inflammation, infection, endometriosis, peritoneal dialysis and cancer, will benefit from our results. In the longer-term, we will have identified biological targets that could be blocked with drugs and new ways to deliver them to prevent adhesions forming in patients. By working with surgeons and industry colleagues, our findings will help to develop new therapies to reduce scar tissue forming in patients having abdominal surgery, with infection or persistent inflammatory disease. Of importance, our discoveries will also be relevant to animals.

Horses, in particular, suffer from adhesions which may cause their intestines to become twisted, and this requires immediate surgery. Therefore, these new therapies will also be of benefit to vets.

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

We collaborate with a number of research groups nationally and globally and present our findings at scientific conferences. Results will be published in relevant open access scientific journals and datasets and tissue banks made available so used by other academic beneficiaries for further data mining and as basis for future studies. Our findings will be of interest to industry and as such, new collaborations will be made in order to move from lab to clinic. In addition, colleagues from the surgical and veterinary community are closely involved in our research and so any new discoveries will be conveyed to a wide audience of end users. Importantly, we participate in public engagement events including science festivals and patient engagement forums to educate, inform and answer questions about our research.

Species and numbers of animals expected to be used

- Mice: 1000

Predicted harms

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

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

We will use adult mice as a part of our research studies as they have similar abdominal anatomy and respond to infection and surgical injury in a similar way to humans by forming adhesions. To better understand the processes involved, we will also use mice that have been genetically altered as this will help us highlight the cells and molecules to target to prevent adhesion formation.

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

Some mice will have an injection of factors into the abdomen that increases inflammation. These factors may include parts of micro-organisms (eg. bacteria and fungi) found in the bowel or chemical irritants present in peritoneal dialysis fluid and known to cause inflammation with internal scarring and possible, adhesion formation. To induce inflammation, mice will receive up to 7 injections of these inflammation-triggering agents into the abdominal cavity using published standard routes and doses. Mice will experience mild, transient pain but should experience no lasting harm from administration of these substances.

Some mice with have abdominal surgery with injury to the bowel and/or inner body wall and introduction of sutures to stitch tissues. In some instances, mice may have abdominal surgery and an injection of inflammation-triggering agent to understand how adhesions may form in a contaminated abdomen. Following surgery, mice will experience some discomfort and mild to moderate pain which will be managed with pain-killers. Animals will be monitored closely and should fully recover after 2-3 days.

Groups of mice that have undergone a procedure may also receive therapeutic substances delivered in a gel or solution to identify key processes involved in adhesion formation. On some occasions, genetically altered mice will be used where a gene of interest will be switched on or off to work out its role in adhesion development. To control the behaviour of the particular gene of interest some animals will be given substances by mouth, injection, or through food, that can target the particular gene and switch it on or off.

Animals will be humanely killed at the end of the study by schedule 1 method. Experiments will typically last for a week and no longer than 28 days as our previous data has found after an inflammatory episode, adhesions remodel over time and are stable by 28 days. The minimum number of procedures a mouse will receive is one and the maximum is four.

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

The mice undergoing abdominal surgery are likely to experience post-operative pain which will be controlled by appropriate anaesthesia and pain-killers and should last no more than 2-3 days. Mice are also expected to experience some weight loss (generally between 10-15%) after surgical procedures, and this should return to pre-operative weight within approximately 5 days. Intra-peritoneal injection of inflammation-inducing agents (on up to 7 occasions) may cause some mild discomfort in mice after each injection but this should be transient. After the procedures, mice will be provided with soft bedding, a mash diet and kept warm. All animals will be closely monitored for any adverse effects and will be humanely killed if approaching severity limit at any point in the study.

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

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

100% moderate - mice that have either undergone abdominal surgery or injections of an inflammation- inducing agent into the abdominal cavity, or both.

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

- Killed

Replacement

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

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

We have teased out some of the key events of internal scarring using cell culture systems in the laboratory. We have also analysed human adhesion scar tissue collected from patients while undergoing repeat surgery. However, more detailed studies looking at how factors, cells and signalling pathways interact are also needed. For instance, blood clots that form between damaged organs after surgery, and ongoing inflammation due to infection, are proposed to be the main triggers of internal scarring and adhesion formation. We can only successfully replicate these events in whole organisms to really understand which blood clotting factors are involved, how inflammatory cells move in and out of the abdomen and ultimately what makes adhesions form. Our mouse models will produce findings that we will be able to relate to patients as they involve mammals rather than non-mammals such as frogs or fish which do not have the same blood clotting or inflammatory systems.

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

We also use non-animal alternatives to help inform our animal studies. These include samples of human abdominal tissue collected from consenting patients undergoing surgery and cells isolated from waste peritoneal dialysis solutions that we can culture in the laboratory. We are also developing a human 3D abdominal wall model in a tissue culture dish that has the same features as the abdominal wall in a patient. This non-animal model will allow us to ask very specific research questions such as how cells communicate with each other and what triggers clotting factors to be produced and blood clots deposited. These studies complement and in part, will replace our animal studies.

Why were they not suitable?

We work alongside many experts including surgeons so that we are sure that we use the most relevant models and experimental techniques to mirror what happens to patients as closely as possible.

Although non-animal alternatives are important to address specific research questions, they can not model the blood and inflammatory systems and anatomy of the abdomen to fully address how internal scars form over time. New developments and alternative models will be continually monitored and changes to experimental plans altered accordingly.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientificobjectives, if any. These 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 work closely with statisticians to ensure accurate calculations are performed to determine the minimum number of animals required to achieve our goals. We have performed these studies previously so have a good understanding of the number of animals required to produce statistically significant findings comparing treatment groups with non-treated groups. In addition, we have reviewed the work of others in the field to help inform our decision on the number of animals required in each group, dose and volume of agents to deliver and relevant controls to use. Calculations typically show that we need group sizes of 6-8 to achieve the quality of results we need. Cell culture studies, then pilot animal studies, will initially be performed for any new treatments using a reduced number of animals to help inform numbers required in each study to produce meaningful data.

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

We ensure that experiments are well planned before starting so that we are in the best position to generate statistically meaningful results. Every experiment has been designed to fulfil the objective of utilising the minimum number of animals that will ensure that statistically significant results are obtained with appropriate controls. NC3Rs' experimental design guidance and experimental design assistant (EDA) is used to inform our experimental design, improve our procedures and guide the statistical analysis of data. Experimental design includes bias avoidance strategies such as ensuring that animals are

randomly assigned to different groups, surgery performed by the same individual for each study, endpoints analysed by researchers blinded to treatment and where measurements are subjective (for example adhesion scores, ultrastructural analysis), researchers will again be blinded to the treatment received. Mice will be house together and we will avoid gender bias by including both male and female animals at equal ratios and use genetically-altered mice including mice with normal behaviour or expression of the target gene (wild-type) as well as those with half the expression of the target gene (heterogenous) or no expression of the target gene (homogeneous).

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 get as much information as possible from each animal by collecting multiple samples – blood, fluid, tissues, cells - to be analysed. As similar cells exist, tissues and fluids may also be collected from the thoracic cavity as well as the abdominal cavity, in order to generate a tissue bank/resource for future studies and to share with other groups. In this way, we will gain a greater knowledge of the workings of whole body systems such as how body cavities communicate with each other by the movement of inflammatory cells and signalling factors. In addition, we will culture mouse diaphragms in the laboratory and these will replace some live animal studies. One diaphragm will be bisected, and one half used for the treatment arm and the other for the control so further reducing the number of animals required. As such studies using new therapeutic agents will initially be performed using the cultured diaphragm or new 3D human abdominal wall model before the most promising treatments are tested in vivo so refining the live animal studies performed.

Refinement

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

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

Adhesion formation in humans can be caused by injury triggers such as surgical trauma, foreign bodies like sutures, lack of blood flow (ischemia) as well as bacterial/fungal infection. At present, it is not clear whether all these triggers drive the same processes or signalling pathways in abdominal cells that lead to mature adhesion formation. We will investigate internal scarring and adhesion formation using two mouse model systems that we have previously used; the first represents inflammation- induced scarring based on the effect of chemical irritants or dead pathogens or components of them (Protocol 1) whereas the second represents surgery-induced scarring (Protocol 2) with inflammation driven by abrasion injury and the use of sutures. Scarring is known to be more extensive following surgery and infection and so in some cases, an infection-driven inflammatory trigger will be combined with a surgical injury

Surgery-induced internal scarring involves injury to the surface of the inner abdominal wall by abrasion injury or restricting blood flow to a limited area, with or without abrasion injury

to the bowel. Sutures are used at the site of trauma and to close the abdomen as would occur in patients undergoing surgery.

The degree of injury is controlled using a tailor-made instrument to deliver a reproducible injury to the surface of the inner abdominal wall. Sutures are used that produce minimal foreign body reaction as in humans. Mice recover well from the surgery with pain relief provided and although adhesions form, these procedures do not lead to complications such as bowel obstruction or any lasting harm.

Inflammation-induced abdominal scarring involves injection of chemical irritants such as dialysis fluid and/ or dead or parts of microorganisms such as lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria or zymosan (a cell wall component of fungi) and/or other pro-scarring factors into the abdominal cavity. Commercially available inflammation-inducing agents will be used to ensure correct dosage, sterility and purity of products to generate sterile peritonitis, rather than using live organisms.

To control the expression of a particular gene of interest in GA animals, some animals will be given substances by mouth, injection, or through food that can control that particular gene and switch it on or off. This helps us to work out how adhesions form by telling us what role that particular gene plays in the scarring process. We may also use this method to express genes that colour code cells so we can track them as they move to the injury site or to delete specific cell types to see if they contribute to adhesion development.

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

Adhesion formation involves inflammation and blood clot formation between closely packed damaged abdominal organs and inner abdominal wall. Young mice do not have a fully formed immune system and do not have the same make-up of blood clots so would not be suitable alternatives. In addition, non-mammalian animals do not have the same anatomy, show the same type of immune cells and their clotting system is different from humans so not appropriate to use. The models described are well established, produce consistent results and findings have been disseminated in our publications. We will use tissue from culled mice in order to set up diaphragm organ cultures for experiments to study how abdominal cells communicate with each other and respond to specific triggers of adhesion formation.

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

We have extensive expertise already using these mouse models under our previous licences and will make several refinements based on our experience. These include re-grouping mice a day after surgery rather than keeping them individually housed. Welfare checks will be continuous throughout the studies and adverse effects such as weight loss, change in behaviour and pain scores reviewed at least daily using set template forms. We will explore the use of hydrogels as a means of slowly releasing therapeutic factors in the abdomen over time. We previously used Brewer’s thioglycolate to induce a mild inflammatory response with only transient adhesions as well as heat-inactivated Cryptosporidium parvum (a gut parasite) to produce a more robust inflammatory response over several weeks with adhesions maturing 3-4 weeks post-injection. Using commercially available inflammation triggering factors such as LPS and zymosan as alternatives, we will improve reproducibility, standardisation and dosage. These agents have been used previously by many groups to induce experimental sterile inflammation in mice.

We work alongside many experts in their field including surgeons, vets and industrial colleagues so that we are sure to use the most relevant models and experimental techniques to mirror what happens to patients as closely as possible. New developments and alternative models will be continually monitored and changes to experimental plan altered accordingly.

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

We will follow published guidelines such as the PREPARE guidelines: http://journals.sagepub.com/doi/full/10.1177/0023677217724823 and :https://norecopa.no/prepare to ensure we plan and conduct studies in the most refined way. Other resources will be reviewed regularly including those from the NC3Rs and other published guidelines. In addition, local recommendations will be monitored via the animal facility newsletter, training opportunities and workshops.

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

We will regularly review the NC3Rs news and advice and are signed up to receive the NC3Rs newsletter. We will discuss our studies with the NC3Rs Programme Manager and attend scheduled workshops. We will also review tissue engineering and surgery websites, databases and publications to remain informed of any developments in the adhesion research field.

37. Directed Axonal Regeneration for Biohybrid Interfaces

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

Advanced prosthesis use, Biohybrid electronic interface, Regenerative peripheral nerve interface, Axonal regeneration, Flexible electrode array

Animal types	Life stages
Rats	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this research is to understand how electrical stimulation can be used to enhance and guide peripheral nerve repair and re-growth (regeneration) . Specifically, it will study which electrical treatments (known as stimulation regimes) can direct regrowth into specific areas of target tissues, enhancing the degree of nerve branching. Ultimately this will lead to an understanding of how to create more effective connections between the nerve ending and implanted electronic devices (known as electrode arrays) used to control robotic artificial limbs.

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

Why is it important to undertake this work?

Over the past 20 years, patients have been observed to stop using their advanced prosthetic limbs, preferencing simpler prosthetic limbs that are purely mechanically driven with a body harness and pulley system. This decision to replace the advanced prosthetic limb with a simpler device is called advanced prosthetic limb abandonment. One of the most common reasons for advanced prosthetic limb abandonment, especially in robotic upper limbs, has been a lack of reliable functionality when undertaking daily tasks. Reviews on both upper and lower robotic limbs have identified two characteristics that lead to reduced function: limited fine movement control (dexterity) and the inability to adequately perceive touch. Both of these current limitations can be addressed by increasing the number of connections between the damaged nerve and the sensors which control the limb, known as electrodes. Our work investigates the performance of a new construct which integrates the damaged nerve with spare muscle tissue and electrodes. Known as a biohybrid interface this approach enables greater numbers of electrodes to interact with the nerve, which is expected to help robotic limb users have greater dexterity and improved touch sensation, reducing advanced prosthetic limb abandonment and improving patient quality of life.

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

Our work to produce connections between damaged nerves and electrode arrays, known in the field as regenerative peripheral nerve interfaces (RPNIs) with improved targeting of specific parts of the nerve, defined as spatial selectivity , has the ability to improve patient outcomes for a number of neural conditions. New electrode array designs with holes that facilitate nutrient flow to the tissues can allow devices to be put into the body (implanted) at the same time as amputation as opposed to the current practice of doing two surgeries to preserve tissue health. Electrical stimulation provided during the nerve regeneration period can increase the growth of nerve processes called axons, which further increases connections between the device and tissue. These technologies also enable new high-resolution tissue-electrode interfaces, which could restore patient function to levels beyond what is currently possible.

We expect there to be 2-3 papers on the impact of the electrode arrays on nerve regeneration and spatial selectivity alone; however, with the development of advanced electrode arrays (with more connections to the nerve) and connection with different types of body tissues, this will likely increase significantly, with the potential to have outputs that include new electrode designs, further funding and new applications for implantable electrode arrays.

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

In the short term, we will demonstrate the impact of various electrode designs on axonal regeneration when implanted at the time of the RPNI construction (in one surgery). These results will have a direct impact on clinical decisions for timing of electrode implantation when using the RPNI as a bioelectronic interface in amputees.

In the medium (less than 5 years) term, we will determine how useful multiple recording and stimulation sites are on a single RPNI construct for the purpose of improved limb control and feeling in prosthetic limb users. These results will influence the specific design of the electrode array chosen for implantation.

In the long term (more than 10 years) the benefits of tissue-electrode interfaces with high spatial selectivity will be fundamental to the field of neurotechnology and neurocience as a whole. This should be feasible based on our published work in flexible electrode array production and our collaborative relationship with two groups of plastic surgeons.

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

This work will develop understanding and data about the tissue-electrode interface. The biological response of these electrode arrays will drive new projects within our own research group and within our collaborators' labs. We expect our developments to be extended by our collaborators who will also begin incorporating our electrodes, into existing research on interfacing with various tissue types to encourage regeneration of nerve fibres.

We are also planning a publication in a plastic surgery research journal with our collaborators that will communicate the results of these studies to the clinicians who make the decisions of when to implant and how many surgeries. Unsuccessful approaches are paramount to identify in these publications to inform the clinicians of previously attempted work without the need for further assessment unless future work reveals otherwise.

Species and numbers of animals expected to be used

- Rats: 168

Predicted harms

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

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

We are using the small animal, rodent model in this project because they are one of the most common small animals used in peripheral nerve regeneration studies, so there is a greater knowledge base to which to compare. A rat model of this surgical construct will provide the smallest mammalian model that is feasible to recreate the axonal regeneration environment based on the size of the nerves, muscle grafts, and electrodes. It is sufficiently large and similar to humans in its immune response and axonal regeneration mechanisms.

We are using adult rats because it is known that the regeneration of peripheral nerves is much greater in adolescence, so we want to assess axonal regeneration during the adult phase of life to avoid any confounding factors. We are using both sexes to avoid any sex-based bias and a limitations of applicable results.

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

All rats in experimental groups will undergo a surgical procedure in which a muscle in the lower limb responsible for extension of the toes is surgically removed and reimplanted around the end of a branch of the main leg nerve (the sciatic nerve). This is an established surgery which replicates the cutting of nerves undertaken during amputations in humans.

The rat is put under anaesthesia and kept under for the duration of the surgery. A cut is made in the skin of the lower limb to access a specific muscle called the extensor digitorum longus (EDL). The EDL is taken from the body and reimplanted higher up in the thigh. A branch of the main sciatic nerve is cut and embedded into the muscle belly of the EDL, which is then anchored to the bone with stitches. The muscle provides the nerve with healthy tissue to grow into and can also be used as a site to place electrodes that can record signals from the nerves (replicating the recording needed to control robotic limbs in humans).

After securing an electrode array on top of the muscle, the connecting wires are threaded up the back of the rat by creating a tunnel just under the skin. The wires are left unconnected, inside a capped port that is implanted in the skin of the rat's upper back. The surrounding skin grows around the port, but allows the wires to be safely exposed and connected to electronic hardware during defined periods across the study timeframe.

The animal is allowed to recover from this procedure and the cut nerve grows into the EDL muscle for up to 12 weeks. During this period of regeneration, the electrode arrays in the animals will be used to stimulate the growing nerve (a process which is expected to improve the nerve growth) through the skin embedded port, every day up to a maximum of 21 days. The stimulation is applied through a tethered connection within the port and will be for up to 3 hours per day. Recordings will also be taken through this same port during the entire regeneration period. When both recording and stimulation sessions occur on the same day, both procedures will be completed during one session of up to 4 hours in duration.

At the end of the 12 weeks, the animals are put under terminal anaesthesia and regrowth of the nerve is tested by placing an additional recording electrode further back towards the sensory processing centre of the brain. The electrode implanted on the EDL muscle is then used to stimulate the regrown nerve, with the degree of activation assessed through the recording electrode. Anaesthesia will be carefully monitored and increased if necessary, during the stimulation protocols. The animals will be humanely killed at the end of this study.

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

The primary adverse affect is expected to come from the lack of the muscle that extends the toes in the rat. The rat will develop an irregular walking pattern (gait) to compensate for the inability to lift its toes during its normal stride. The irregular gait will not interfere with regular day to day activities of the rat and we are not expecting any lifestyle changes for the rat. The irregular gait will endure for the entire 12 week period, but rats will learn to compensate during first two weeks based on previous studies.

The rat will be carefully monitored for damage to the top of the toes that will come into contact with the ground more often than normal. We do not expect these issues, but if there is a breaking of the skin on top of the toes or ulceration, the animals will be humanely killed to avoid undue suffering.

During electrical stimulation , we will not exceed the amount of charge required for muscle contraction while the animal is conscious. Pain indications will be carefully and consistently

monitored by at least two observers during these stimulation sessions and if a pain threshold is reached, the stimulation session will end, and pain thresholds will not be exceeded in future sessions.

Post-operative acute (5-10%) weight loss can be observed for approximately 1 - 3 days. Any observation of <15% weight loss will be treated with daily weight monitoring and feeding of softened foods.

For 5% of animals, surgical wound complications such as local inflammation, itching, redness, soreness, swelling, or infections may be observed throughout the study. If any surgical wound complications are observed, topical creams and/or systemic antibiotic treatment will be administered as required post-operatively under the advice of the named veterinary surgeon (NVS) and the named animal care and welfare officer (NACWO).

Animals will be housed for up to 4 hours individually during the recording and stimulation sessions. As animals will normally be housed in pairs at all other times, this may cause mild stress to the animal. We will use environment enrichment to ease the stress on 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 expected severities for all operations are moderate. All animals will experience the same muscle graft surgery that is the primary source of the adverse effects, so 100% of the animals fall under this moderate severity. Only 50% of the rats will receive stimulation protocols, so only half the rats could experience any adverse pain effects from electrical stimulation. We do not expect anything more than single-instance, mild pain based on the protocols we have in place.

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

- Killed

Replacement

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

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

The main goal of this project is to evaluate the biological integration of an electrode array with a clinically implemented surgical construct and assess the impact of external stimulation from this array on the axonal regeneration within the surgical construct. We have already assessed the effect of chronic implantation on a rat nerve with this electrode array design and have now paired the electrode array with a specific clinical application. A rat model of this surgical construct will provide the smallest mammalian model that is feasible to recreate the axonal regeneration environment based on the size of the nerves,

muscle grafts, and electrodes. It is sufficiently large and similar to humans in its immune response and axonal regeneration mechanisms. Although extensive lab based testing has been carried out to optimise several aspects of electrode array, the study of complex processes in the body such as inflammation, scarring, and peripheral nerve regeneration requires assessment in an animal model. This is mainly because lab based tests cannot reproduce the multitude of interactions that occur between different cells and tissues in a living organism, which are key determinants of the efficacy and performance of the biohybrid tissue-device construct.

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

Cell line based

Schwann cells

Rat motor neuron cells in combination with a muscle cell line

Primary cell based (cells taken from a human or animal)

Co-culture of dorsal root ganglion neurons and Schwann-like derived stem cells

Explanted tissues harvested from animals

Dorsal root ganglion (cell bodies adjacent to the spinal cord) in a co-culture with neurons and glial cells

Why were they not suitable?

The aims of this study are to evaluate the biological integration of an electrode array with a clinically implemented surgical construct and assess the impact of external stimulation from a specifically designed implantable electrode array on the axonal regeneration within a clinically implemented surgical construct, the RPNI. We are specifically investigating the degree of axonal branching within the RPNI and the molecular mechanisms that encourage further branching under external stimulation. Any cell culture models or tissues are not similar enough to a body to recreate the axonal regeneration environment needed to inform clinical decisions.

Cell line based

Signalling pathways that are critical for complete axonal regeneration are not present in simple cell line based models for regeneration. These models are too far from the native axonal regeneration environment in the human body.

Primary cell based

While more essential signal pathways exist in primary cell cultures, the 3D environment of axonal regeneration does not exist in a culture model, which severely limits the ability to determine the amount of axonal regeneration occurring in the RPNI, which is a primary outcome of this study to translate to clinical decisions within the practice of plastic surgery collaborators.

Explanted tissue - ex vivo based

While whole tissues outside of a body are typically suitable for creating a body like environment for axonal regeneration, they do not survive on the time scales needed to observe regeneration into a muscle graft. They are limited to only 2-3 weeks, with reduced axonal regenerative capabilities after the first few days.

Reduction

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

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

The number of animals needed for this project is based on similar implantation studies carried out previously by our group and by others in the literature. This estimation considers the number of experiments, experimental groups, and animals per group that are needed to detect significant differences in the variables of interest, consistent with the aims of our study. Apart from the animals needed for the experiments to test our research hypothesis, this number also accounts for animals needed to carry out initial pilot experiments for the optimisation of the surgical workflow. In addition, we have taken into consideration the sample size suggested by relevant international standards such as the ISO 10993-6, which specifies methods for the assessment of the local effects after implantation of medical devices.

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

We will strive to select the optimal experimental design to guarantee the scientific validity of our results while using the minimum number of animals possible. We will also take steps to ensure that the experiments we perform are unbiased and have statistical power to ensure that the study will yield quantitative results. For instance, the allocation of the animals receiving either the electrode implants or the control RPNIs, as well as the order in which the surgeries will be performed will be randomised using a random number generator. Where possible, the processing of the samples retrieved for assessment will be blinded to reduce bias. In addition, data from the pilot experiments will be used to confirm the parameters for the design of the main study. Lastly, we will use the NC3R's Experimental Design Assistant (EDA) to carry out and improve the design of our study, as well as the analysis of the data and the reporting of our findings.

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

Overall, we will aim to minimise error by ensuring adequate training of the staff and that all surgical equipment and infrastructure is in optimal conditions. Based on our experience, the initial pilot will provide valuable insight for the optimisation of every step of the experimental workflow and to identify any unforeseen contingencies prior to the main

study. We will continue to work in parallel to develop more sophisticated computer models of the RPNI with repaired nerves so that stimulation parameters can be optimized for the spatial selectivity study to reduce the number of groups of animals we need to use. We will also thoroughly test the engineered electrode constructs to ensure optimal performance before we test them in animals. Lastly, we will coordinate with other researchers to maximise the use of tissues and organs from the animals after humanely killing.

Refinement

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

Which animal models and methods 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 the most widely used models for nerve damage and regeneration studies, because of their similarity to human biology. Adult rats are the preferred choice for this study because their larger body parts will enable surgical procedures that would be impossible on a smaller sized mouse, which will also minimise error and prevent unnecessary pain and suffering. Rats have also been selected due to their extensive use in the literature as models to assess axonal regeneration and long- term electrode array implantations, which includes studies performed by our own group. Anesthetic protocols have been refined and will be undertaken by trained and competent personnel to ensure that homeostasis, body temperature and depth are maintained. We (and our collaborators) not only have previous experience undertaking this type of work, but several peer-reviewed studies are available for benchmarking and refinement of our experimental approach.

Cutting of only a single branch of the sciatic nerve to implant into the muscle graft allows for significant improvements in mobility of the rat hindlimb compared to cutting the entire sciatic nerve. During all surgical procedures, pain relief will be used. Following recovery, recording and stimulation regimes will be carried out on awake animals via the skin-embedded port mounted on the back of the rat using a tethered connection. As confirmed by collaborators in the field, the back-mounted skin port eliminates the surgical risks that come with a rigid, skull-mounted port. Tethering will be carried out using a cable attached to a swivel system that is anchored to the lid of the cage, with a length that allows free movement within the chamber. Lastly, animals will be housed in pairs wherever possible. However, they may be temporarily housed individually for up to 4 hours during recording and/or stimulation in order to prevent interactions between the 2 animals that would interfere with the electrical connections

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

Rodents are the simplest animal models that are compatible with our research aims. The use of less sentient or immature animals is not a viable option for this project due to the need for mammalian nervous and immune systems that are suitable for axonal regeneration studies over extended timelines. Additionally, mice are too small for the

surgical procedures and subsequent analysis of the tissue, where larger cellular structures and tissue sections are required to assess the nerve regeneration.

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

Overall, we will strive to follow the best possible practice for all experiments carried out in this project with a strong emphasis on those involving live animals. Pain and discomfort will be minimised using sterile technique, as well as post-operation monitoring and care, including pain relief. Animals will be routinely and carefully monitored after surgery by us and animal facility staff. Actions to mitigate adverse effects will be taken as advised by the veterinarian and other animal facility staff. Where any advice can be integrated into future procedures as a refined protocol, this will be undertaken in accordance with the NC3Rs strategy for improving animal welfare and quality science. Particular observation will occur on the impacted hindlimb following the construction of the RPNI. Rats will be housed in cages with soft bedding to reduce the impact of a rough surface on the bottom of the cage on the paw. Moreover, to ensure the behavioural needs of the animals are met, rats will be housed in stable pairs where possible and institutional best practice will be followed with regards to provision of environmental enrichment and other aspects of animal care. Our experimental approach will allow us to perform repeated measurements to maximise the amount of information each animal provides, while minimising the number of animals needed for the study. Similarly, we will strive to select the most refined methodology for recording and stimulation in order to minimise animal distress throughout the duration of the study. Furthermore, computer studies will continue to be run in parallel to ensure a constant refinement of the experimental parameters used in this study.

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

We will follow all relevant guidelines available from the National Centre for the Replacement Refinement and Reduction of Animals in Research (NC3Rs), the Laboratory Animal Science

Association (LASA), the report from the Joint Working Group on Refinement (JWGR), Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE), and Animal Research: Reporting of In Vivo Experiments (ARRIVE).

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

We will constantly revise our experimental strategy to reduce, refine, and replace the use of animals based on data produced by our own group and by staying up to date regarding current advancements in the field. All staff involved in this license will attend relevant meetings held by our institution and other third parties related to recent advances in the 3Rs and their implementation. We will stay up to date with information and resources from external sources including the National Centre for Replacement Refinement and Reduction of Animals in Research resources (NC3Rs, https://www.nc3rs.org.uk/), as well as the 3Rs group at our establishment. We will also maintain close communication with personnel doing daily maintenance and overseeing the facility to ensure that all possible

measures are in place to preserve the welfare of our animals. Lab meetings will routinely be used to discuss the latest techniques and advancements in 3Rs for our animal studies.

38. Targeting galectins and their binding ligands in cancer (renewal)

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

Galectins, Tumour growth, Cancer metastasis, Galectin inhibitors, Small molecular weight inhibitors

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 aims of the proposed investigations are 1) to test the influence of galectin family members, their interaction with binding partners on tumour growth and metastatic spreading and 2) to assess the effectiveness of novel galectin inhibitors, alone or in combination with other therapeutic agents, on inhibition of tumour growth and metastasis in mouse models. These studies will help to increase our understanding of the mechanisms underpinning tumour growth and spread and aid the development of novel therapeutic drugs for treatment of cancer and metastasis.

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

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

Why is it important to undertake this work?

Cancer is the commonest cause of death in the UK, accounting for one out of every four deaths with a total of more than 150,000 deaths each year. Despite enormous effort and investment, effective treatment of this disease remains a big challenge. It is increasingly believed that therapies targeted at specific molecules rather than the classic chemotherapy approaches, hold the key for more effective cancer treatments in future. As a complex disease, cancer is regulated at multiple levels by various molecules during the process of tumour formation and development. One group of such molecules is a family of closely-related sugar binding proteins called galectins which are increasingly shown to be critical in cancer growth, development, progression and spreading by multiple mechanisms. Targeting the actions of galectins is increasingly considered an attractive therapeutic strategy to improve cancer survival. Our research has revealed several galectin-binding proteins that are involved in galectin- mediated cancer/metastasis promotion. Our recent studies have also identified several novel galectin- binding inhibitors from natural (dietary) and synthetic sources which show to effectively inhibit galectin-mediated cancer cell behaviours in the lab in cultured cells.

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

This project will aid the translational development of several novel galectin inhibitors recently identified in my lab. These novel galectin inhibitors are currently being filed for patent protection. An establishment spin-out is also in discussion of formation to lead the development of those novel galectin inhibitors as galectin-targeted novel therapeutic drugs for treatment of cancer. Data obtained from this study will support the formation of the university spin out and will aid the essential pre-clinical investigations and development of our novel galectin inhibitors before early phase clinical trials. The results of this project will be published in peer-reviewed journals after IP evaluation by the university IP team.

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

This programme will include testing the effectiveness of several novel galectin inhibitors recently identified in my lab on inhibition of tumour growth and metastatic spreading of common cancer types such as colorectal, breast and pancreatic cancer and melanoma. The social and economic impact of finding new drugs that can effectively target these common cancers is huge. The information obtained from this programme will also help the scientific community to develop new therapeutic strategies for more effective treatment of cancer.

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

Once the data obtained from this study is evaluated and properly protected for intellectual property, they will be made public by presentation to national and international conferences, by publication at peer-reviewed journals. All publications will be disseminated through the normal scientific pathways as well as via social media platforms such as Linked, Facebook and twitter to maximise the dissemination and impact of the discoveries.

Species and numbers of animals expected to be used

- Mice: 1800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 selected mice as our model species in this study because it is the most common and most established in vivo models in cancer research. Testing in mice is the gold standard in assessing effect of new agents on tumour growth and metastasis for development of new therapeutic drugs. Currently, thre is no other better in vivo models in the fields. The mice will be typically 6~8 week old. This life stage of mice is in mature age with good immune function and best resembles condition of human adults.

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

In this programme, the mice are typically subjected to injection of tumour cells. This is followed by injection of the testing inhibitors periodically for several weeks. The tumour growth will be monitored closely by measuring the tumour size or by imaging.

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

All mice will be monitored closely throughout the experiment procedures. The experiment mice will experience momentary needle stick pain at the tumour injection stage. With development of the injected tumour, mice may experience weight loss or poor health such as failure to respond to gentle pinch, loss of consciousness, laboured breath, difficulty of movement, or diarrhoea when the mice will be killed humanely with the fully established standard protocols. In some experiments, the grafted tumours will be removed by surgery after the mice are put into sleep. A very small number of mice may suffer from wound infection after surgery. If this results in ill health such as the symptoms described above, or at the end of each experiment, the animals will be humanely killed with the standard protocols and the tissues will be taken for further analysis.

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

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

The mice will experience mild severity at the stage of injection of tumour cells and compound inhibitors while mice will experience moderate severity if the step of tumour removal by surgery is included in the procedure (optional step). With development of the grafted tumour, a small proportion of mice (<10%) may show weight loss or poor health which would fall into moderate severity branding. When grafted tumours are removed by moderate surgical procedures with sutures under general anaesthesia with recovery, the wounds in some animals (<10%) may not heal completely and infection or suture rapture

may occur. Occurrence of metastasis in some animals in long period may also result in weight loss of poor health. These animals will experience moderate level of severity. All mice will be monitored closely throughout the procedures. At the end of every procedure, all animals will be killed humanely and the tissues will be taken for further analysis. Considering both the mandatory and optional steps in the protocol, overall, approximately 80% of the mice will suffer from mild and 20% moderate severity in 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?

Adequate testing in animals is essential to help us to understand cancer. For development of new anti- cancer drugs, currently there is no alternatively but to first test them in animals, most commonly in mice, before any clinical trial in human.

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

Cancer cell lines

Why were they not suitable?

Whilst testing the cancer cells in petri dish in the lab helps to gain information at molecular level in understanding the diseases, they are not able to recapitulate the complex macro- and micro- environment in the body that has a huge impact on tumour development.

Reduction

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

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

We will maximally use non-animal methods such as culturing cells in petri dishes in the lab to identify the optimum dose of the testing inhibitors before conducting any test in animals. The comprehensive use of in vitro experiments will allow us to only test the most effective inhibitors at the optimum dose in animals to maximally reduce the use of animals. Furthermore, we will use mathematical models to calculate and predict how many animals will be needed for each experiments. This will also help us to use a minimal number of

animals to achieve the study objectives (https://www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda).

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

Our comprehensive use of non-animal methods will allow us to only test the most effective inhibitors at the optimum dose in animals to maximally reduce the use of animals. For design of each animal experiment, we will use mathematical models to first calculate and predict how many animals will be needed for each experiments. This will also help us to use a minimal number of animals to achieve the study objectives. We will also use the assistance of EDA online tool to help to ensure the use of minimum number of animals consistent with their scientific objectives, methods to reduce subjective bias, and appropriate statistical analysis.

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

In addition to the above measures, we will also conduct thorough literature search and consult with our animal service unit in the experiment design stage for any other consideration and possibility to optimise the number of animals in each experiment.

Refinement

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

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

Mouse model will be used in this study because it is the most common and most established animal model in cancer research. The mouse model is also the gold standard in testing new anti-cancer drugs before they can be tested in human. Inoculation of tumour cells will be done by injection in the study which can avoid any other dramatic inoculation procedures such as surgery to reduce pain, distress and to the animals. Testing substances will be administrated orally or by injection (dependent on the aims of the testing to as close as to future clinical application) which will cause the least distress and pain to the animals.

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

We have selected mice as our model specie in this programme because it is the most common and most established animal model in cancer research. The mouse model is also the gold standard in testing new anti-cancer drugs before they can be tested in human. Currently there are no better in vivo models in the field.

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

We will constantly analyse our experimental data and also review new literature reports during the study in order to refine our protocols (such as the use of dosage, routes, period and schedule of substance administration). This will allow us to use the minimal concentrations of the testing substance and the optimum methods to achieve the best while cause minimal harm to the animals and use minimal number of animals.

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

We are aware of a number of good reporting guidelines. For example, the PREPARE guideline by AJ Smith et al, Lab Animals 2017, 52,135-41; the BJC guideline by Workman et al, Guidelines for the welfare and use of animals in cancer research. Br J Cancer, 2010, 102, 1555-57.

In this study, the NC3Rs "Responsibility in the use of animals in bioscience research" guideline will be strictly followed.

https://www.nc3rs.org.uk/sites/default/files/2022-01/Responsibility%20in%20the%20use%20of%20animals%20in%20bioscience%20research%202019. pdf

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

We will keep the communication channel open (emails, phone numbers) all the time. We have an internal animal welfare group, whose membership includes our institution animal welfare officer. We are always kept informed and updated of any advances in the 3Rs from the regulatory body though our institutional animal welfare officer and our animal welfare group. We will apply any new requirement or regulation from the regulatory body, when it occurs, accordingly and immediately to our experiments during the study.

39. Understanding motor neuron degeneration and regeneration

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Motor neuron, Drug development, Repair, Degeneration

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

In the childhood motor neuron disease spinal muscular atrophy (SMA), the cells which connect the spinal cord and muscle known as motor neurons, break down. The aim of this project is to better understand how and why motor neurons break down, and find ways to encourage repair.

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

Why is it important to undertake this work?

If left untreated, children affected by Spinal Muscular Atrophy have a very poor prognosis, with most not expected to reach their second birthday. New treatments are now available which have a big impact upon the most severely affected patients. The treatments allow patients to live for much longer, but often they are living with chronic and life altering

disability. It is important that we continue to develop new treatments which can protect motor neurons and help them repair the damage which has been done.

SMA is very much at the forefront of research. It is the only neurodegenerative disease with a viable treatment. For this reason, the questions facing the SMA field, such as how to repair damage to motor neurons which occurs during disease, will be of huge relevance to other neurodegenerative diseases.

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

This project has the potential to identify new ways of slowing the breakdown of motor neurons and helping them repair. This will produce valuable new knowledge and data sets which can be exploited by other researchers. Outputs from this work also include publication and presentations at conferences. Possible drug identification would also lead to patents being filed and new collaborations with pharmaceutical companies to move to clinical testing. In the long term this could lead to new medicines to help improve prognosis in SMA and other neurodegenerative diseases.

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

As our project progresses and we share results with other researchers via publication and presentation, this can help similar work going on in other labs, and help accelerate progress.

The development of clinical trials has the potential to improve outcomes for patients, and our ultimate goal is to reduce the severity of the symptoms experienced by patients and enhance their quality of life.

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

A portion of this work is being performed as part of a collaboration between ourselves and 2 other groups. The charity funding this work remain active participants and are really good at linking us up with the most relevant people who can help progress and solve problems. We also attend 2-3 conferences per year and present data as both talks and posters, and engage and discuss with other researchers, drug companies and affected patients. All work will be published in open access journals. All resources will be shared with other researchers.

Species and numbers of animals expected to be used

- Mice: about 4000 over the project duration.

Predicted harms

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

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

This project uses mice. Many of them are genetically manipulated to have similar genetic defects as patients with SMA. Others are genetically manipulated with synthetic genes which allow us to better understand how motor neurons repair themselves. Although motor neurons can be grown in a dish, when this is done the cells lack all the normal connections which they make with other cell types. Since we are looking at the process of breakdown, followed by repair, it is exceptionally difficult to mimic this scenario using cells in a dish. The mouse models used to study SMA have been shown to be highly predictive of the human condition, and have been absolutely instrumental in developing all the drugs available to date.

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

Many of the animals used in this study will be used for breeding, and producing the mice with the desired genetic problems. The mice with the right genetic combination will show symptoms of the disease. We will observe them carefully during their life span, then kill them in a humane way, and take tissues which we can analyse. Often we need to look at an age before symptoms start so these mice are killed in a humane manor before they experience any symptoms.

Some of the mice will also be treated with drugs which are similar to those which the patients receive. These are delivered either by getting the mice to drink the solution or delivered via an injection. Some of these injections are done into the periteoneal cavity, so the injection will go into the abdomen. For some injections, they are into the ventricles of the brain, so the mice will be put under general anaesthetic, then a small needle is pushed through the skull to deliver the drug to the correct place, then the mouse wakes up and recovers from the procedure.

We might test how this affects the ability of the mice to move, by getting them to climb up a wire grid, or putting them on their back and seeing if they can turn over.

Many of the mice carry synthetic genes which have absolutely no effect on them, but allows us to analyse their tissues in different ways. For these mice, they are kept in normal cages until they reach the desired age (usually 2-4 months old). They then receive an oral dose of a drug called tamoxifen, which has no detrimental effects at the doses that we use but induces changes in their genetics which allows us to study a particular process or make the mice better so we can study recovery. We then kill them using a humane method and remove tissues for analysis.

For some mice we will perform a nerve injury. For these experiments, the mouse is put under general anaesthetic and a small hole in the skin is made. The nerve is located and forceps are used to compress it for about 30 sections. The wound is closed using glue and the mouse left to recover. It will be given some pain killers to help with discomfort but mice usually recover very well.

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

Most of the mice we use don't have anything wrong with them, or are killed before they suffer any symptoms, so they don't suffer any pain or distress. The mice which are designed to be models of the disease and reach symptomatic ages will have some difficulty walking and climbing. As they get older they might have some difficulty feeding

and breathing. When it gets to this point they would be humanely killed. Some mice will also require daily injections which can be uncomfortable at the point of administration but cause no lasting harm and they recover quickly. However where necessary i.e. injections into the brain, we do injections under general anaesthetic. We also use oral dosing routes when possible. For mice who undergo surgery to perform a nerve crush, the procedure is done under general anaesthetic and pain killers are given after the operation. They may have some weakness in 1 leg, but it does not affect the ability to walk, climb or feed.

Expected severity categories and the 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 mice will be classed as subthreshold (83%). Some mice will be classed as mild (around 3%) and some as moderate (around 16%).

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 studying how motor neurons break down and how they repair. These cells do not function in isolation, and rather only operate normally when in their native environment surrounded by all the other cells types which they are connected to. We also administer drugs to the mice to mimic what these drugs do in patients. There are lots of factor which will influence where the drug goes and how long it takes to work, which cannot be mimicked in cell culture systems.

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

There are huge advances in cell culture systems, where the connections made between neurons and muscle can be recreated between muscle cells and nerve cells grown in a dish. These model systems are improving all the time and recent reports suggest that neuromuscular junctions between muscle and nerve can be formed in a dish. There have also been reports of defects in the electrical properties of the neurons and the number of connections that they make with the muscle when using cells which carry mutations associated with Amyotrophic Lateral Sclerosis (ALS).

Why were they not suitable?

Although great advances have been made, these systems are immature. We have shown that developmental age has a massive impact upon regenerative and degenerative processes, so it is important to examine a life stage similar to that of the human patient.

These systems are also comparatively simple, compared to the complexity of the connections within an organism. For example, although initial formation of neuromuscular junctions is very much driven by the nerve interacting with the muscle, during regeneration, cells called schwann cells direct re-growth and formation of new connections. These Schwann cells can myelinate axons and guide regeneration. So far, there are no culture systems which have been able to incorporate schwann cells alongside motor neurons and muscle. It is also becoming apparent that not all motor neurons are alike, and there are subclasses which show different properties in disease, and this is a really important aspect of disease which we need to model. Since we don't understand what makes different motor neurons unique, we cannot yet model it in a dish. It is important to note that all the things that we will learn from our animal studies will help improve in vitro models and they are likely to become better in the future because of 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?

Around 80% will have genetic alterations which are not harmful. Of the 20% which will carry mutations which could make them have symptoms similar to the human disease.

This number is based upon our previous experience of similar previous work and is based upon our annual returns from a previous licence. We have considered the predicted proportion of mice which are likely to have the correct genotype to be experimental mice, based upon our current breeding strategies. We have also considered the need to optimise some procedures in pilot experiments to make sure they are working exactly as expected so that we do not perform experiments on large numbers of mice which are not giving valuable data. Experimental groups sizes are set based upon our previous experience to give sufficient numbers to be able to detect meaningful changes. Variability is reduced by using in-bred mouse lines which have high genetic similarity. Often many tissues can be taken from a single mouse which maximises the data produced.

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

All experiments are designed so that we have enough animals to make firm conclusions at the end of the experiment, but not more than we need. For all the experiments designed to test drugs we do so in a way in which we have 1 control group and multiple test drugs all of which get compared to the control group. This reduces the number of control 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 often run multiple experiments in parallel so that a range of tissues can be taken from 1 animal and used for different purposes and projects. For example, if a collaborator is looking at the same drug but their interest is in a different tissue, then we share tissue between labs to avoid repeating the experiment.

Refinement

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

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

In this project we will use mouse models of spinal muscular atrophy which have been genetically manipulated to reflect the human condition. These models have been widely used and been shown to be highly predictive of the human condition, and have been used to develop the 3 approved drugs for this disease. Selecting the best model reduces long term harm as the data produced is valid and translatable and ultimately reduces the numbers of mice required in the future.

Affected mice have a short lifespan and display a phenotype for around 8 days. Initially this phenotype is very mild, but may cause some distress towards the last 2 or 3 days, in that the mouse might have some difficulty walking or climbing. However, as these mice are very young, they are still being fed by their mother so they don't need to climb to access food. When we observe a 15% weight loss, or see that that mouse can no longer feed independantly, it is euthanised. Mice are checked regularly, which includes twice daily checks when the mice are approaching later stages of disease. Our time points are limited by the phenotype of the mouse, but untreated mice have a maximum life span of 12 or 18 days, depending on the specific line.

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

Our focus is on neuromuscular junctions (NMJs). Non-mammalian NMJs (e.g. zebrafish or drosophila) are not good research models. They are structurally really different and use different chemicals to communicate with the muscle. Their ability to regenerate is also really different, having a much higher regenerative capacity. Therefore, if we want to understand regeneration in a mammalian system, and what limits it, then we have to use a mammalian model. We also want to look at stress put upon motor neurons over a prolonged period for some experiments. Given that the lifespan is so short for less sentient animals, this is not possible.

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

We will use oral gavage wherever possible instead of intraperitoneal injection. We will use good practise when using injections, changing the needle each time. We will administer local and general anaesthetic whenever appropriate.
We will ensure staff are trained to a high level.

We will employ the least stressful method of euthanasia.

Where a mouse has difficulty climbing, food will be provided on the base of the cage. Animals will be regularly monitored for any adverse effects.

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

We will follow the PREPARE guidelines.

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

We regularly check information on the NC3Rs website and engage with all the local training and updates which are provided.

40. Understanding the impact of healthy and compromised pregnancy in mother and offspring

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

Pregnancy, Developmental programming, Metabolic health, Placenta, Endocrine programming

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 determine the molecular mechanisms which control how nutrients are transferred from mother to offspring in the reproductive period, and understand how the failure of this process (either due to genetic or environmental causes) impacts the lifelong health of both generations.

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

Why is it important to undertake this work?

Fetal programming of life-long health is a well-established phenomenon which describes how poor growth of the baby is associated with increased risk of metabolic, endocrine and cardiovascular disease in later life. However, we still do not understand how these processes are linked, and without mechanistic insight, we cannot understand how the consequence of poor baby growth might be mitigated or even reversed. Further, we do not currently know how to break the perpetual cycle of adversity and ill-health from one generation to the next which is thus contributing to the continuing rise in common comorbidities.

Extremes of birth weight at term are not only correlated with health problems in early life, but also predisposes to metabolic disorders in adulthood. As rates of obesity and diabetes increase in the general population, the outcomes of pregnancy have worsened and maternal and fetal health remains a significant public health issue. Despite the enormous cost to public health of compromised pregnancy, little is known about the normal process of nutrient transfer between mother and baby, and how this process can be affected by both genetic and maternal lifestyle factors.

Our project seeks to explore mechanisms that link growth and metabolism in adult life – by exploring how the mother mobilises her energy and nutrient stores for transfer to the developing offspring. We will use this knowledge to further understand the genetic pathways in the mother and the offspring that control nutrient transfer, and how these can be affected by maternal diet. Finally, we will assess the long-term consequences of poor nutrient mobilisation for both mother and baby, and test interventions for their ability to reverse a poor early life environment.
What outputs do you think you will see at the end of this project? New information:

1) We will discover how the mother makes nutrients (especially lipids/fats) available to the fetus during pregnancy by releasing them from her own stores in adipose tissue in a timely manner. 2) We will understand the mechanism by which some of these molecules get transported to the developing baby across the placental barrier. 3) By manipulating the maternal diet we will understand how obesity and a nutrient-poor lifestyle can prevent/impair the transfer of essential nutrients. 4) We hope to discover some of the hormonal signals that the baby produces to tell the mother to increase her nutrient release. 5) By supplementing the mother with nutrients in specific periods we hope to understand the most effective interventions to reverse a compromised maternal lifestyle.

We will also generate new methodologies that other researchers can use to track fats and adipose stores during pregnancy and in disease contexts.

Publications:

All of our findings will be published in open-access, peer reviewed journals.
Who or what will benefit from these outputs, and how? Short-term outputs:
New information will benefit the academic community by increasing our understanding of nutrient flow in pregnancy. In addition to publication of our data we will deposit large datasets in publicly-available repositories where they are accessible to other scientists working on common and diverse biological problems.

We will publish new methodologies that allow researchers to i) track adipose tissue dynamics and ii) track metabolites in real time and in pregnant animals. These techniques can be applied to a range of health research problems.

Outputs beyond the project:

Ultimately we will collaborate with clinical colleagues working in the areas of nutrition and Women and Children's Health to evaluate our findings in human populations where pregnancy is compromised. Our research will suggest new mechanisms by which a poor diet compromises pregnancy outcome, and determine time windows and specific interventions that can improve the long-term health of mother and offspring. Our genetic studies will also allow personalised medicine by identifying individuals who may be particularly vulnerable to nutrient deficiencies and require more monitoring/intervention during the reproductive period.

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

The PI is an active participant in the UK Endocrinology, Developmental Biology and Metabolism communities, serving on scientific panels and organising scientific meetings in this area. Consequently the group has wide dissemination of work beyond the process of publication, and is poised to implement new collaborations.

The host institution has a strong Department of Women and Child's Health, with ongoing clinical studies. Here discovery scientists working with animal models interact with colleagues with a strong translational component to their work, allowing rapid transfer of ideas from research lab to clinical cohort studies of pregnancy and early life interventions.

High quality negative data will be published in open access journals and in data repositories to prevent unnecessary replication by colleagues in the field.

Species and numbers of animals expected to be used

- Mice: 13000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 model organism of choice for studies in which mammalian gene function is addressed. It remains the most tractable mammalian organism for mutation and manipulation of genetic material and for subsequent phenotypic analysis. Furthermore, laboratory mice are usually genetically homozygous therefore genetic background effects that complicate analysis and interpretation of outcome are minimised, and fewer animals need be used to achieve statistical significance. The mouse genome has been extensively sequenced and characterised, and protocols for embryo and reproductive manipulations are well-established and safe for the mouse. By definition, placentation and viviparity is specific to mammals therefore non-mammalian models cannot be used.

Since we are studying the impact of compromised pregnancy across the lifecourse of both mother and offspring, animals at multiple life stages must be used: Embryo, Neonate, Juvenile, Adult, Pregnant adult.

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

Typically, genetically modified animals without any harmful phenotype will be bred. Female animals will then be used as mothers to investigate metabolic and hormonal pathways in pregnancy and lactation. Some of these animals will receive modified diets, or genetic manipulations will be induced by injection/inclusion in the food of a drug. Over the reproductive period (around 19 days pregnancy with an additional 21 days lactation) the mother may receive a series of metabolic tests, including injections of glucose and insulin, or a small amount of blood may be drawn for testing, and she may be anaethetised for imaging. The mother will usually be humanely killed at a set time point in pregnancy or the lactation period, and data from embryos/pups collected. In some cases either the mother or the pups may be kept for a further period/into adulthood and receive metabolic testing as above to determine the long term consequences of a disrupted pregnancy.

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

Most of our genetic or dietary modifications are expected to produce no adverse effects. However, in some cases we will generate new modifications that have unanticipated adverse phenotypes. In these cases we will monitor all newly produced animals from novel strains/condition and immediately humanely kill any animals demonstrating such phenotypes. We will then modify our experimental plan so these animals are not required or an earlier/non-harmful timepoint can be used.

Some of our protocols require animals to experience food withdrawal. This will lead to transient weight loss and mild discomfort.

Animals will experience transient pain when injected with substances.

Expected severity categories and the 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: 67% (most animals under the breeding protocol with no harmful phenotype)

Mild: 21% (animals in the metabolic protocols experiencing mild discomfort due to injections/small volume of blood withdrawal)

Moderate: 12% (animals in the metabolic protocols experiencing moderate discomfort due to combination of food withdrawal in pregnancy and transient administration of substances by injection).

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 and lactation are complex whole-system processes that link the actions of placental and maternal hormones to the metabolic behaviour of central and peripheral tissues. We intend to use the mouse as a model organism for the experiments outlined in this project. The mouse is an excellent model for mammalian physiology, and as a placental mammal is an appropriate system in which to study interactions between mother and fetus. Since we wish to study whole body energy homeostasis during pregnancy there is no alternative but to use a live mammal. Moreover, both placentation and metabolic physiology are well studied in the mouse, and parallels can be drawn between this study and a significant body of previous work. Both humans and mice have a hemochorial placenta, so we believe any finding in the mouse can be extrapolated to understanding human health and disease.

However, we are aware that there are differences between the mouse and human regarding the length and relative rates of gestation, thus our data will ultimately require validation in human cohort studies. We are currently working with colleagues in the KCL Department of Women and Child’s Health to design experiments that test the predictions of hypotheses generated in the mouse in human cohort data.

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

Cell culture models

In order to test hypothesis where we are interested in the molecular response of a specific cell type to a stimulus - e.g. the response of an immature fat cell to a hormone, we employ the use of immortalised cell lines. These reagents have utility in providing mechanistic insights into one aspect of a whole-body response, with significant caveats (see below).

Organ-on-a-chip

To study the response of maternal tissues to the pregnancy-induced hormonal milieu we considered using 'organ-on-a-chip' technologies. This is a strong approach because the system to some extent replicates complex cell-cell interactions found in metabolic tissues.

Why were they not suitable?

Cell culture models

Experiments utilising cell culture models have been instrumental in elucidating the molecular mechanisms that lead to the differentiation of metabolic tissues (such as adipose and liver), the placenta and brain from progenitors. However, it is increasing appreciated within the field that in-vitro models do not fully mimic the behaviour of these

tissues in vivo, since the tissues: i) contain multiple interacting cell types and ii) reside in a broader neuroendocrine milieu that is crucial for normal function.

Organ-on-a-chip

To our knowledge there is not yet a suitable system for the study of metabolic organs in pregnancy.

Reduction

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

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

Numbers have been estimated in 2 ways:

To determine numbers of animals we need to breed to generate experimental animals of required genotypes, we used previous experience of animal use over the course of the past licence.

For the experimental animals we estimate based on previous use and calculations included in grant applications that fund the research into the future.

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

In our experimental design we carry out robust statistical analysis of all data and ensure that our results are of the highest quality. We aim to generate litters with both controls and experimental genotypes represented, allowing us to control for inter-litter variation and to use pairwise/repeated measures statistics, thus increasing the power of the analysis. All dams/experimental animals will receive the same treatment and be housed at similar stocking density. Researchers will be blinded to genotype while performing tissue collection and cell counting/stereological analysis. Our stereological protocols use randomisation to ensure that no systematic biases are introduced at the sampling/measurement stage. Most analyses performed at least 2 independent cohorts of animals and generate overlapping data thus providing replication. In addition we will usually perform the analyses on both sexes and we expect to see concordance between the sexes for most experimental outcomes.

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

To ensure the minimum number of animals we take four approaches: 1) We rigorously monitor the breeding regimen of our stocks generating animals from the fewest breeding pairs/trios and increasing these only as experiments require. 2) The team shares animal material between several projects and communicate our animal needs during group

meetings to increase the efficiency of work and minimise wastage. This also includes keeping track of stored samples and distributing them, rather than generating new samples from additional animals. 3) We generate and freeze primary cell lines from animals and use these for mechanistic and exploratory studies prior to embarking on an animal experiment. 4) We collaborate extensively with colleagues within and outside London and worldwide, and share animals, tissues, cells and samples with others authorized to use them. This means that others do not need to generate new mutants or samples, minimising animal numbers. We have benefitted from others sharing samples with us.

Refinement

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

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

We will use 2 broad classes of mouse model in this project.

Firstly we will use female mice that have been genetically manipulated to have blocks on their ability to generate or store nutrients, or are subject to modified diets such as high fat or high sugar. Since we are interested in whether these mice can successfully transfer nutrients to their offspring, the dams themselves will generally be viable and healthy (since they must reach reproductive age).

Secondly we will generate mouse crosses where the offspring have mutations in genetic pathways that affect the placenta or nutrient transport in embryonic/neonatal tissues. In this case we will collect data from timepoints where any defects are first manifested in the offspring or mother, and in many cases this will be before the animals are born, thus minimising suffering.

Our methods will evaluate how well the mice are able to store and metabolise nutrients, and respond to hormones. These protocols are broadly based on human clinical measurements (such as glucose tolerance tests), and generate no lasting harm to the animal.

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

The mouse is the model organism of choice for studies in which mammalian gene function is addressed. It remains the most tractable mammalian organism for mutation and manipulation of genetic material and for subsequent phenotypic analysis. Furthermore, laboratory mice are usually genetically homozygous therefore genetic background effects that complicate analysis and interpretation of outcome are minimised, and fewer animals need be used to achieve statistical significance. The mouse genome has been extensively sequenced and characterised, and protocols for embryo and reproductive manipulations

are well-established and safe for the mouse. By definition, placentation and viviparity is specific to mammals therefore non-mammalian models cannot be used.

We will measure metabolic outcomes over the course of pregnancy, arising from processes in both the mother and the offspring, therefore terminally anaesthesised animals cannot be used to generate longitudinal data.

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

We predict from previous studies that over-expression/deletion of candidate genes or regulatory factors are unlikely to cause severe phenotypes. We will use established reagents and protocols hence minimising the unknown effects on the mice. When possible, inducible or tissue-specific constructs will be used so mice will not display a phenotype until candidate gene expression or deletion is induced.

Here we will generate and use mice that are defective in genes regulating pathways important in the control of growth and pregnancy, and conduct studies to understand how they work to affect the health and wellbeing of the mice and their offspring. Protocols include metabolic analyses and tests of hormone production, and following the growth and development of the mutant mouse. The majority of these procedures (>95%) are expected to cause minimal adverse effects (mild or less). In a minority of cases the animal may experience moderate severity (<5%), for example occasional hypoglycaemia as a result of insulin tolerance tests. Some genetic modifications may cause phenotypes with mild severity, such as changes in body weight or appetite regulation. These animals will be closely monitored to minimise secondary adverse effects. At the end of the experimental protocols animals will be killed by humane methods.

We will minimise the chances of unwanted, severe outcomes from genetic manipulation by testing outcomes at early stages of the process; in cell culture and in very young embryos.

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

Where possible and if required we will use published protocols and consult expert colleagues for on- hands training. We will keep to the recommended best practice in accordance to NC3Rs, following the PREPARE and ARRIVE guidance when planning new experiments and reporting the outcome of studies.

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

The institution has an excellent Biological Services team who regularly communicate workshops and guidance from the NC3Rs. The PI and researchers will regularly attend these events to remain up to date.

The PI currently sits as a member of the Panel of Experts for the UKRI - BBSRC funding panel. This role includes assessment of animal use in research grants and therefore a good way to remain apprised of current best practice. In addition, panel members receive additional training from the NC3Rs as part of the role.

41. Assessment of abuse potential and evaluation of novel entities to treat substance abuse.

Project duration

5 years 0 months

Project purpose

- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Pharmaceuticals, Abuse, Rodents, Safety, Efficacy

Animal types	Life stages
Rats	adult, juvenile

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To determine the abuse potential of novel pharmaceuticals and to study the effects of experimental treatments for substance abuse 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?

Psychoactive substance abuse poses a significant threat to health and the social and economic fabric of families, communities and nations. In an attempt to limit and control the drug abuse potential of novel drug candidates entering the market place, specific preclinical studies are mandated by the world regulatory authorities (e.g. European Medicines Agency EMA and USA Food and Drug Administration FDA) for all novel compounds in development which enter into the CNS, irrespective of their therapeutic indication. In addition, novel drugs for the treatment of substance abuse are required urgently.

The overall project aim is to provide highly specialised preclinical services to the pharmaceutical and biotech industry to evaluate the abuse liability of drugs which enter into the CNS irrespective of their therapeutic focus and evaluate the efficacy of novel drugs for the treatment of substance abuse. This is achieved by the following three objectives:

1. To assess the abuse liability of novel drug candidates in development which enter the CNS regardless of therapeutic indication, as mandated by the regulators. This includes determination of the pharmacokinetics of the drug candidate, as required by the regulators, so the exposure in the rodent model can be related to the human exposure. These studies will allow the regulator to determine if the drug candidate should be scheduled (yes or no) and if yes what schedule it should be placed into thereby limiting its distribution in the wider population.

2. To assess the abuse liability of new chemical entities in preclinical development for abuse liability. These studies will enable clients to determine if their new chemical entity is advantaged over existing therapies and hence worthy of further preclinical and clinical development.

3. To assess the efficacy of new chemical entities to treat drug abuse. These studies will enable clients to determine if their new chemical entity is worthy of further preclinical and ultimately clinical development.

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

The main output from this project licence will be Regulatory Abuse Liability packages in the form of comprehensive, audited Study Reports. These will be used by sponsors as part of their Regulatory

Submissions in support of the licencing of new drugs.

Studies may also be carried out at earlier stages of the drug development process in order to assist sponsors with an assessment as to whether their novel molecules, or drug targets are likely to have abuse liability issues.

In addition, some studies under this licence will examine the potential of novel pharmaceuticals as treatments for substance abuse disorders, i.e., drug addictions, potentially leading towards the development of new therapies.

Work performed under this licence may be published in the scientific literature, or presented at conferences.

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

To assist sponsors to develop, perform and report a pre-clinical Regulatory Abuse Liability package which will meet the world-wide regulators' requirements, and provide the appropriate scientific support throughout the process. This will allow the sponsor to move rapidly through the clinical development programme without the assessment of abuse potential becoming a rate limiting factor. In addition, these studies will allow the regulators to decide whether a drug candidate should be scheduled or not and if scheduled which schedule the drug candidate should be placed into thereby limiting its distribution to the wider population. In addition, to allow clients to make decisions in regard to their novel

compounds in preclinical development. Does the compound show reduced potential for abuse potential over a marketed product or compound in clinical development? Does the novel compound exhibit efficacy in a model predictive of the ability to treated substance abuse. The medium benefit is the discovery of compounds with the propensity for reduced abuse potential or to treat substance abuse and the long term benefit (likely to be subsequent to completion of the licence) may be a clinically effective drug (since regulatory agencies expect a drug's sponsor to have screened the new molecule for pharmacological activity, prior to assessing its therapeutic potential in humans).

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

Where client confidentiality allows, work performed under this licence may be published in the scientific literature, or presented at conferences.

Early stage studies in particular are collaborative in nature, being run as part of a sponsor's drug development programme. Data and best practices are exchanged in written reports and in regular meetings.

Species and numbers of animals expected to be used

- Rats: 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.

Adult rats will be used for the studies under this licence. The rat has been chosen since its anatomy, physiology, behaviour and genetics has been well-documented. Rats display many of the behavioural and cognitive characteristics of humans and are easier to train than mice and, therefore, they are typically used in the more complex behavioural models, such as those included in this project licence. There is a large literature detailing the use of rats in these abuse models and methods proposed in this licence have all been validated in these species. The long-term nature of the studies necessitates the use of adult animals.

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

Rats will be kept in cages either in groups or, when scientifically justified, singly. They will have free access to water and, in most studies food. In some long term studies, rats will have their food intake restricted to approximately 80% of their normal intake. The main reason for this is to improve the animals' health as long-term free access to food can lead to obesity and related health conditions.

In all studies rats may be dosed by oral, intravenous, subcutaneous or intraperitoneal routes. Dosing requires a brief period of restraint and may cause mild, transient discomfort.

In some studies, rats will be dosed, typically by the oral route, and then have their body temperatures measured using a rectal thermometer, followed by a period of behavioural

observations. Body weights, and the food and water intake of these rats will be measured daily

Other studies involve the dosing of rats then withdrawal of blood samples from the tail vein at a range of timepoints afterwards (up to 24h).

Three studies under this licence require rats to be trained to press levers within operant chambers into which they are placed for between 10 minutes and 4 hours per day on 5-7 days of the week. Two studies requires surgical implantation of intravenous catheters which are exteriorised at a port on the animal's back. Surgery is carried out under general anaesthesia and rats are administered analgesics to reduce postoperative pain. The intravenous port is connected to an infusion line protected by a spring tether for typically 2h per day, but on occasion up to 4h per day. Compounds of interest are dosed via the infusion line.

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

The majority of the animals are likely to have a to experience only brief periods of mild discomfort, generally on a daily basis. The majority of studies will involve the administration of drugs which enter the central nervous system (predominantly orally or via an indwelling catheter) for behavioural testing. Drug treatment might be once or repeated. Some compounds will have been extensively evaluated prior to assessment. In such circumstances no adverse effects are expected. Some compounds may not have been tested extensively in animals and unexpected toxic effects might arise which could cause pain, suffering, lasting harm or in extreme cases death if humane end points were not applied.

Some studies involve general anaesthesia, with an associated low risk of harm or death, as in humans. The animal models employed may involve training in specialised equipment which may produce transient discomfort/stress. Upon completion of procedures animals will be 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)?

Rat, 8% mild, 92% moderate.

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

- Killed

Replacement

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

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

The project objectives each require investigation of candidate substances to be tested in an integrated behavioural/physiological/pharmacological model that requires the whole animal which cannot be replaced by in vitro or ex vivo studies. Prior to testing, it is expected that candidate substances will have been selected on the basis of extensive efficacy and safety evaluation (compounds assessed as part of the abuse liability regulatory package) or in silico, in vitro, ex vivo and in vivo experiments for more novel compounds undergoing evaluation prior to moving into development.

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

Non-animal alternatives are unsuitable for studies under this licence. Regulatory guidelines require the assessment of compounds in animal models.

Why were they not suitable?

The in vivo Abuse Liability package is a requirement of Regulatory bodies worldwide. The acceptable studies to be included in Regulatory submissions are clearly defined within published guidelines, and are largely non-negotiable.

Reduction

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

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

The estimated animal usage is based upon a figure of 2000 rats per year over 5 years, sufficient to run approximately 6 abuse liability packages and 3 substance use disorder discovery phase studies. The exact number of animals used will be dependent upon external factors such as the number of clients and the success of those clients in designing suitable drugs. Additionally all studies are designed on an individual basis dependent upon the scientific questions being asked, so use differing numbers of animals.

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

1) The expertise of the company's statisticians who are able to perform power calculations to ensure that studies are suitably powered to detect the difference of interest. We also have a large historical data set that we can draw upon for this purpose.

2) Use of animals where appropriate to evaluate multiple test compounds or reinforcing drugs (e.g. heroin, cocaine, etc.). This not only reduces the number of animals used but has the following advantages: allows comparison of effects of related drugs in the same animals (including pharmacokinetic evaluation), minimises the number of animals that need training in a particular task, and surgical intervention.

3) When evaluating drugs that potentially possess sedative euphoriant properties, the rats need to be trained to self-administer a positive control. Opiates and opioids, e.g. heroin, remifentanil and oxycodone, are often used as the positive control when training

rats to self-administer opioids. These drugs cross the blood-brain barrier very easily and very quickly, and consequently, are highly rewarding (reinforcing) which means that a large proportion of the rats given access to these drugs develop strong self-administration responding. Morphine is not an ideal choice as a training reinforcer because it does not cross the blood-brain barrier quickly and it has relatively poor brain penetration. Thus, in comparison with compounds like heroin, remifentanil and oxycodone, morphine has much less rewarding effect. Additionally, some rats find the effects of morphine aversive, particularly at higher doses. Therefore, it takes more sessions to train rats to self-administer morphine and the proportion of rats in an experimental study that achieve successful acquisition of drug-self-administration is much lower (40-50% typical responder rate with morphine, 80-90% typical responder rates with heroin).

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

We make extensive use of pilot studies where a small group of animals (typically 3) is run ahead of the main study. This allows doses of test compound to be optimised and adverse effects minimised during the main study phase.

Another approach that we take to optimise doses prior to committing a large number of animals to a study is to run pharmacokinetic experiments in advance and use the data from these to set groups for the main studies.

In some cases, results from pilot and pharmacokinetic studies facilitate a decision to no longer proceed with further work in larger groups of animals.

Refinement

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

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

The rat has been chosen since its anatomy, physiology, behaviour and genetics has been well- documented. Rats display many of the behavioural and cognitive characteristics of humans and are easier to train than mice and, therefore, they are typically used in the more complex behavioural models, such as those included in this project licence. There is a large literature detailing the use of rats in these abuse models and methods proposed in this licence have all been validated in these species. Rats are social animals and will be group housed unless single housing is believed to be preferable for the animal’s wellbeing, or the scientific validity of the study.

The models detailed in this project licence have been established, used and refined by the company over the past 15 years. They have all been validated using suitable compounds and widely used by the pharmaceutical industry.

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

The tests to be performed under this project licence require a species that expresses complex behaviours, such as the rat. Less sentient species cannot be trained to perform the operant tasks (e.g. lever pressing), that are required. Similarly, a conscious state is required for the expression of operant behaviours, so anaesthetised animals are unsuitable.

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

Following surgical implantation of jugular vein catheters into animals for IVSA work, a member of staff is designated to monitor the animals throughout their recovery from anaesthesia and for the first few hours after recovery. This allows for rapid intervention in the event of problems arising, including humane culling if necessary. Animals undergoing surgery are pre-treated with an analgesic (e.g.meloxicam), with a second dose being administered on the day following surgery. As IVSA animals are housed singly to avoid damage to their catheter ports being caused by cage mates, rats are allowed to socialise in groups of up to three for an approximately fifteen minute period at the end of their daily experimental sessions.

Rats on IVSA and DD studies are monitored via closed-circuit television while they are in the operant chambers each day. This allows scientists to monitor animal behaviour and welfare, intervening if necessary.

We make extensive use of pharmacokinetic (PK) and pilot experiments prior to committing large numbers of animals to a study. The PK studies allow us to determine that the compound doses that are proposed to be used produce the correct plasma exposures, ensuring against under- or overdosing of rats, the former leading to a possible repeat study, and the latter risking adverse events.

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

When planning studies, we will make reference to the PREPARE and ARRIVE guidelines and checklists. We also utilise the guidelines on the LASA and NC3Rs website with respect to techniques such as dosing, blood sampling and animal handling.

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

Our studies are based within a university animal facility As such, we can discuss 3Rs related changes to techniques with university staff and academic groups. We also work closely with the named persons (NVS, NACWO and NTCO) to ensure that we are familiar with current best practices in in vivo research. We also work collaboratively with study sponsors, many of whom have in house research teams with which we can discuss and share best practices.

We also participate in the university's AWERB system and our project licence applications and amendments are discussed at the regular AWERB meetings, offering the chance to discuss refinements.

As stated above, we have an in-house team of highly experienced statisticians and involve this group at the experimental design stage. This group keeps up-to-date with relevant changes in study planning and analysis.

We regularly monitor relevant scientific literature to stay up-to-date with the abuse-liability field of research. We attend scientific meetings and, where client confidentiality allows, publish on our work.

42. Genetic and environmental analysis of genomic imprinting

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Low birth weight, Maternal anxiety, Maternal depression, Maternal neglect, Behavioural disorders

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 will determine whether changes in the expression of imprinted genes contribute to disease with a focus on low birth weight, maternal mood disorders and behavioural disorders. In addition, we will ask whether prenatal adversities cause changes in the expression of imprinted genes in the fetus and/or placenta, and then use our models to identify interventions that prevent or ameliorate adversity-driven 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?

In the UK 14% of mothers have clinically concerning symptom of depression and 25% have clinically concerning symptom of anxiety - often co-modibidly. Children exposed to a mood disorder in utero are at increased risk of being low birth weight, as young children they are more likely to demonstrate impaired socio-emotional and cognitive development, and as young adults they are more likely to be depressed and anxious. The prevalence of

all these conditions is highest in socio-economically disadvantaged populations and populations under stress. It is not possible to fully explore causality using just human population data.

Our recent experimental studies prove that maternal anxiety and detrimental outcomes for offspring (low birth weight, anxiety) can all result from the same underlying pathological problem, which is placental endocrine insufficiency driven by genetically altering the expression of epigenetically imprinted genes in the placenta (Phlda2, Peg3, Cdkn1c, Ascl2). Moreover, we have some data to suggest that changes in the expression of these genes can be driven by prenatal adversities such a poor maternal diet.

Our work seeks to further understand the relationship between adversity and the miss-expression of these genes, to interrogate the mechanism by which changes in the placenta impact behaviour in mothers and their offspring, and to examine how poor maternal care alone can have detrimental consequences for offspring. Importantly, for this new licence, we wish to test interventions which might ameliorate mood symptoms and break the intergenerational cycle between adversity and mood disorders.

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

Novel models: We will develop new genetic and environmental models to investigate the short and long-term consequences of poor growth and placental dysfunction on maternal and offspring wellbeing.

Novel data: We will generate data to achieve a better understanding of the mechanisms underlying low birth weight, complications of pregnancy including mood disorders, and the fetal programming of behavioural abnormalities in offspring. We will identify factors (genetic/lifestyle) that alter imprinted gene expression and thus influence birth weight, pregnancy, behaviour and/or metabolism. We may identify biomarkers with diagnostic potential.

Dissemination of data: We will generate scientific publications from these data.

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

In the shorter term (0-3 years), our work will benefit scientists studying placental development and maternal adaptations to pregnancy. There has already been a great deal of interest in our findings that imprinted genes respond epigenetically to dietary adversity in pregnancy, and that genetically manipulating the expression of these genes in the placenta can influence the care given to the offspring by the mother evidenced by citations of our work, invitations to present at meetings and news stories in the press.

In the longer term (3-10 years), our work increasing the understanding of adversity-drive disease will benefit clinicians, healthcare workers, human health and well-being. Our work will inform policy-making and influencing public policies benefiting pregnant women and their children.

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

Dissemination of research to the scientific community: We will publish our work in peer reviewed impactful journals, give conference presentations and deposit data in repositories for other scientists to access.

Dissemination of research to clinicians, healthcare workers and the general public: We will continue to engage with clinicians and midwives through workshops and talks. We will engage with the general public by running annual interactive workshops. As a longer term aspiration, we will contribute to further surveys on maternal mental health and translate our findings on placental lactogens from mouse to human pregnancy through our own human pregnancy cohort, through collaborations and new funding initiatives.

Publication of negative data or unsuccessful approaches: Although it can be challenging to get data accepted for publication if the results are not significant, we recognise that it is critically important to inform the community of these results and ensure others do not repeat unnecessary experiments. We will achieve this through use of journals that facilitate this process, such as Plos One.

Collaboration: Collaboration is key to maximize the quality and breath of data obtained from an experiment. We have long held collaborations with scientists in the UK and abroad (USA, Canada, Japan, New Zealand), and we will continue to work with collaborators to ensure the highest quality data can be obtained from our experiments.

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.

Animal use is necessary because the processes we are studying cannot be modelled in any other way. Given the intimate and intricate relationship between the mother, the fetus and the placenta during pregnancy, and the relationship between the mother and her pups after birth, it is not possible to use an in vitro system.

Mice are chosen as our experimental model since their physiology is well studied, they are genetically modifiable and they are the lowest vertebrate group appropriate for studying pregnancy and maternal behaviour.

As we are researching the impact of early life adversity on the health of both the dam and her offspring, we study all stages of development from fertilisation into adulthood, and even across generations.

We use exclusively female mice to study pregnancy and maternal behaviour. However, when we work with genetically and/or environmentally modified models, we include both male and female mice for studies that assess outcomes of an adversity (e.g. assessing behaviour) or to test an intervention to account for sexually dimorphic responses.

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

In order to study pregnancy-related disease and their consequences in mice, our research requires the generation and breeding of genetically altered mice and their wild type siblings either by breeding or through transfer of embryos. Transfer of embryos requires the production of embryos by superovulation which involves hormone injections. Surgery is used to generate vasectomised males and sometimes in the transfer of the embryos into recipient females.

Pregnant and/or lactating females (dams) are used to study behaviours such as those related to maternal anxiety and maternal caregiving. Most tests are short (a few hours) and take place several times over weeks. Sometimes dams are remated in order to study their behaviour in a second pregnancy. Sometimes we study maternal behaviour in combination with an environmental modification (e.g. dietary alteration, exercise, administration of drug substances). Dams can gain or lose weight and we might then undertake insulin and/or glucose tolerance testing to examine glucose management which involves injections and taking tiny amounts of blood. We might also collect blood from pregnant females at 3-4 timepoints during pregnancy to measure pregnancy hormones, very similar to the tests that pregnant women undergo. Dams may undergo non invasive imaging which may occur in combination with the injection of an imaging agent, and under general anaesthesia. Imaging usually takes less than one hour and can be repeated 2-3 times, but not on the same day. In addition to studying maternal behaviour, we are interested in determining the impact of adversity during pregnancy and/or the impact of poor maternal care on the offspring. We therefore study the behaviour and metabolism of male and female offspring using similar approaches to those we apply to dams.

This work may include interventions (e.g. dietary supplementation, exercise, administration of drug substances) aimed at reversing the impact of prenatal adversity.

At the end of an experiment, animals will be killed by a humane method or by perfusion, in order for tissues to be harvested and studied.

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

The greatest adverse effects will be due to a surgical intervention, needle injection and blood sampling.

Genetically altered mice that harbour mild mutations (such a fluorescent tags or luciferase) or with a genetic modification that does not change gene expression should not experience any adverse effects. Animals that harbour mutations associated with loss or gain in gene expression can be born lighter or heavier (±15%) and can have behavioural and/or metabolic dysfunction. For our current lines dysfunction is only detectable through testing and does not cause long term harm. In all cases where we alter the environment, such as by diet, animals may gain or lose weight (±15%) and the can be behavioural and/or metabolic consequences. Again, this is only detectable through testing and does not cause long term harm.

For needle injections and blood sampling transient pain at the sight of injection/sampling is the primary side effect. Mice experience mild, transient pain and no lasting harm.

For surgery, typically, animals recover well from these and no overt impact is observed. In rare cases the animal may die as a result of the anaesthetic or surgical intervention. However, if this happens, a full post-mortem is conducted to establish the cause of death and, if appropriate, protocols can be modified to reduce the likelihood of this occurring. Animals are given long-acting analgesics during the surgery and are monitored for welfare and weight changes for days/weeks post-surgery by the researcher, the animal facility staff and the named vet. Any sign of ill effects and the animals are either treated or removed from the study, in discussion with the Named Veterinary Surgeon (NVS) for advice.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

At most, the procedures used in this Project Licence will be classified as 'moderate'. For behavioural assessments, non aversive testing is preferentially conducted, with minimal aversive testing applied where no other alternative is appropriate. Needle injection, non-invasive imaging and mild genetic modifications are typically classified as mild. Surgical interventions are moderate severity.

In each of these cases, good laboratory procedures have been implemented, in close collaboration with the NVS, to ensure that suffering will be minimal. Moreover, the animals will be monitored very closely for signs of distress/discomfort and removed from the experiment and/or culled if welfare concerns are identified. Many of the procedures/experimental tasks are not expected to have any adverse impact on animal welfare, such as undertaking behavioural tests or using motor or cognitive training interventions.

Depending on the genetic status of the parents, we can have 0% or 50% of a cohort of animals phenotypically altered (by virtue of harbouring a genetic mutation on the silent allele or the active allele, respectively). For our environmental modifications, 100% of the animals are exposed which may be wild-type or genetically modified. Our dietary modifications are classified as mild.

60% sub-threshold

30% mild.

10% 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?

While we do use alternative strategies wherever possible to understand disease (such as cell models), it is not possible to model maternal-offspring interactions and their longer term consequences in a cell culture system. It is also not possible to use a non-mammalian model as pregnancy, placental development and lactation are features of mammals not observed in other vertebrates. Mice are chosen as an experimental model since their physiology is well studied, they are genetically modifiable and they are the lowest vertebrate group appropriate for studying pregnancy.

Which non-animal alternatives did you consider for use in this project?

It is possible to use placental stem cells to explore some aspects of differentiation. It may be possible in the future to model the interface between the placenta and the uterus in a organoid system.

Why were they not suitable?

The hormonal, physiological and behavioural interactions between dams, placentae and fetuses cannot be modelled in a culture system nor can we study the consequences of exposure to adversity on maternal and offspring 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?

These estimates are based on (1) using data from my previous research to predict future use and (2) calculations associated with ongoing and anticipated grant applications from my research group.

Experience: Under our previous licence we have used ~3000 mice. We plan similar work going forward and estimate a similar number of mice will be required under the new licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When designing experiments, we rigorously attempt to balance the use of animals with the requirement to design a well-powered study that is capable of robustly addressing the scientific question. We will use the NC3Rs Experimental Design Assistant to help us. In many experiments we use a combined behaviour scoring system. This combines results across a number of tasks which can uncover subtle phenotypes with fewer mice. This reduces the number of animals required. In many of our experiments we will collect both behavioural data and harvest tissues and/or serum. We are therefore able to gather lots of information from the same animal, reducing the numbers of animals that we need overall.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All protocols are designed for maximum sensitivity, and experiments are designed to maximise power to detect significant results with the smallest numbers of animals achievable. We seek to reduce the use of animals by undertaking detailed reviews of the literature prior to any work, by noting the number of mice used for similar work by ourselves and other researchers and, where the model and/or intervention and/or strain background is novel, by undertaking pilot experiments to determine the size and variability of the phenotype. This way, we use the smallest number of mice that still allows sufficient sensitivity to detect differences that results from the genetic and/or environmental modification. Additionally we breed and then maintain our genetically modified lines on strain-specific genetic backgrounds. By excluding genetic background effects, we can identify subtle phenotypes with fewer mice. Where possible we breed our lines homozygously. We cryopreserve lines when not in use. We try to maintain all our breeding lines in a single facility so that lines are not duplicated.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 genetically and/or environmentally modified mice. Some of our genetically modified lines show no phenotype as they are reporter lines or there is no change in gene expression. Some of our genetically modified lines show a mild phenotype of low birth weight. Our protocols have been designed to cause the least pain and suffering through minimal use of invasive techniques (e.g. longitudinal non invasive imaging) and use of pain killers when we do invasive techniques e.g. surgery.

Why can’t you use animals that are less sentient?

Only mammals undergo pregnancy with the growth of their offspring in utero followed by maternal care and the provision of milk. As we are studying placental hormones and maternal behaviour, we cannot use any other system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Environmental enrichment is provided for all animals which can include transparent tubes and/or cardboard tubes, chew sticks and the avoidance of singly housed animals where possible.

In behavioural tests we will utilise positive reinforcement (reward).

Where possible we will chose minimally invasive procedures. For example, we study glucose homeostasis by blood sampling from the tail tip rather than the hyperinsulinemic-euglycemic clamp approach, as the latter requires surgery under general anaesthetic. For vasectomy, the vas deferens is generally exposed by the scrotal approach which is less invasive than via laparotomy.

When we do undertake surgery, this is done aseptically in accordance with the most refined standard conditions - currently detailed by LASA and RSPCA. Peri-operative analgesia is given and maintained after surgery for as long as is necessary to alleviate pain. Mice are given post-operative checks for 30 minutes after regaining full consciousness, 3 hours post-op, and then daily for up to 7 days.

In our experiments we use a cumulative score sheet (example provided) to ensure no animal experiences more that a specified number of interventions. We use welfare score sheet (example provided) to check animal welfare when in an experiment. We take advice for the local Named Animal Care and Welfare Officer when necessary. Animals that reach the listed humane end points will be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow best practice guidelines issued from the NC3Rs and Laboratory Animal Science Association (LASA). We will follow ARRIVE and PREPARE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have an Animal Welfare and Research Panel which oversees all work relating to animal welfare. We receive regular, comprehensive update on advances in the 3Rs including the latest news from our local NC3Rs representative. In addition to these emails, we attend workshops and symposia run by our local Biological Services and Standards staff and by our local NC3Rs Regional Programme Manager.
Finally, I also engage heavily with the literature in my research area and follow the development of new models and refined methods for creating these models.

43. Pre-clinical Evaluation of Therapeutic Agents in Rodent Models

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, pre-clinical, rodent models, efficacy

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 primary objectives of this project are:

(i) To develop patient relevant pre-clinical rodent models.

(ii) Use rodent models to evaluate therapeutic agents (mainly anti-cancer) to support progression of effective treatments to human trials, ultimately resulting in validated clinical therapy.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is a broad group of diseases involving unregulated and uncontrolled cell growth that leads to the development of malignant tumours that can invade nearby parts of the body and/or spread to distant organs. There are over 200 different known types of human cancer, which can be treated with different approaches including surgery, chemotherapy, radiotherapy, targeted agents and more recently immunotherapy. Cancer remains a huge health problem globally and specifically within the UK results in just over one in four deaths (2021) and accounts for 167,142 deaths per annum (2017-2019). The latest statistics on cancer mortality from Cancer Research UK (http://www.cancerresearchuk.org) confirm that the major killers remain the solid epithelial cancer types where death is linked to the spread of cancer to other organs such as brain, bone, liver and lungs.

Although several anti-cancer drugs enter clinical trials each year, only 5% of new anti-cancer agents showing preclinical activity go on to be licensed after successful Phase III trials. This is because the pre-clinical models used to mimic cancer do not accurately reflect each of the individual factors that contribute to disease for example the structure of the organ that the cancer originates in, the blood supply to the organ and the immune system influences on the cancer. For these reasons, pre-clinical animal models often lead to unacceptably high failure rates in human clinical trials with around 77% of 800 anti-cancer drugs failing early on in clinic, mainly due to the poor response to the anti-cancer agents in the patients tested.

This data highlights the growing need for Pharmaceutical, Biotech and academia to develop more disease and patient relevant pre-clinical cancer models that better mimic what happens during the growth and progression of cancer in humans, before the new generation of anti-cancer agents enters clinic. This is especially vital with the increased investment into this field and the growing catalogue of new immunotherapeutics, combination treatments and re-purposing of drug candidates.

What outputs do you think you will see at the end of this project?

In order to benefit the clients/collaborators who develop the anticancer agents and ultimately patients who are treated with these agents, the development of pre-clinical cancer models that exhibit greater patient relevance are used to allow these novel agents to be tested in more relevant conditions where environmental factors such blood supply, spatial arrangement, interaction with supporting cells and structures will be better represented. These models require expertise of surgery in some cases as well as generating the cells as well as the use of measuring technology to capture the right data and analysis, which is not readily available in most institutions and companies. These models along with other supporting experiments (e.g tolerability, pharmacokinetic) will enable decision on moving programmes forward into clinical trials or in some cases this may result in a specific anticancer programme being cancelled which may seem a negative benefit, but identifying anticancer agents that are either ineffective or unsuitable for further development can be considered a positive benefit in the longer term as it prevents the unnecessary progression of ineffective therapies to early phase clinical trials and allows the redirection of resources and patients to other projects. Once validated, all models are added to the proprietary databases; access to which is free to all users, as well as abstract submission to national and international scientific conferences.

Who or what will benefit from these outputs, and how?

We are a contract research organization (CRO) that develops/generates and validates animal models for use in client-commissioned studies to evaluate candidate anti-cancer therapies. Our company is engaged in this research by refining and developing pre-clinical models that exhibit greater patient relevance and performing studies with these models in order to support the clients/collaborators we work with who are actively developing new agents and regimens to target cancer. Having established a growing global customer base for the pre-clinical evaluation of anti-cancer agents, we will continue to provide our centralised service to a large number of clients who either do not have the facilities (e.g. virtual Biotech companies), relevant cancer models, expertise or capacity to carry out studies.

Customers of this type have a preference to outsource this work to a 3rd party service provider, where there is a high level of experience, knowledge and clinically relevant models within facilities that are appropriate for such work. In addition to this, our service offering allows customers to focus on other components of their research thus helping them realise benefits more quickly by increasing efficiency and productivity.

Once these animal models are validated they are immediately available for client services and all animal models that are developed at our company are added to our company databases. Access to these databases is free for to all users, so one of the immediate benefits of the animal model development process is that model data, including growth and response to known treatments, key features of the tumour, is freely available to the scientific community, which makes these databases extremely powerful tools for research.

How will you look to maximise the outputs of this work?

Information around new animal models, as well as factsheets and application notes on specific research areas, are freely available to the general scientific community through focused marketing activities including free symposiums with our company and client’s presentations.

Our company is very proactive in attendance at relevant national and international scientific conferences, and we regularly participates in these conferences. Outcome of research is actively shared with the global scientific community through abstract submission at these conferences. In addition, research is submitted to peer-reviewed journals, where possible, in order to communicate findings.

In some cases, cost sharing development of new animal models for clients, or refinements of existing animal models, enable further research and development of more clinically-relevant models. This allows the new models to be available to wider scientific community and findings communicated.

Species and numbers of animals expected to be used

- Mice: 69500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

An intact and developed mammalian model is needed to accurately mimic tumour development and spread. Rodents are the lowest sentient species in which tumours can be grown and developed into models for testing new cancer treatments. Better models of cancer are generated when the cancer cells are directly placed in the organ of origin (e.g. liver cancer cells implanted into the liver of the animal). This mirrors better what will happen in a cancer patient; the tumours grow in a single organ but the presence of an intact mammalian body system may allow the cancer cells to move to other areas of the body (e.g. via the blood stream).

Rodents are readily available in many genetically modified forms so they offer the opportunity to develop very specific tumour models that mimic cancer in humans.

Typically, what will be done to an animal used in your project?

Animal Care & Welfare - All animals will be socially housed in commercially available individually ventilated cages with appropriate bedding material, nesting material and various other items (e.g. chew block, shelter etc..) to enrich their home and stimulate normal behaviours. Food and water will be available at all times, unless there is an experimental need to remove food for up to 16h prior to dosing. All animals will be checked a minimum of daily to monitor their health and welfare. Animals are not expected to experience any pain, distress or lasting harm from their home environment.

Protocol 1 - Under general anaesthetic a needle will be inserted through the chest directly in to the heart and a blood sample withdrawn, immediately followed by death being confirmed by a humane method of killing.

Protocol 2 - Naïve animals will be given vehicle and/or test substances, either alone or in combinations. Typically, mice will be given test substances daily for up to two weeks by one or two routes (e.g. oral dose and/or a dose given into the blood stream); on rare occasions by a maximum of three routes. At the end of the study, animals will be killed by a humane method.

Protocol 3 - For studies looking at how drugs may be distributed and excreted by animals, naïve animals will be given vehicle and/or test substances, as described in the protocol 2 paragraph above. Blood samples may be taken from alive animals but the amount will not cause any pain, suffering, distress or lasting harm. At the end of the study, animals may have a further blood sample taken under general anaesthetic and animals will not be recovered from this anaesthetic but will be humanely killed.

Some drugs have a fluorescent/bioluminescent label and for some studies we may want to find out where this drug goes once injected into the mouse. In these studies, mice can have tumours but do not always have tumours and they are dosed with the labelled drug prior to undergoing an anesthetic event and taking a non-invasive image of the animal using a commercially available imaging machine. The animal will then be recovered and may have further samples taken during the course of the study e.g. small volumes of blood. At the end of the study, animals may have a further blood sample taken under general anaesthetic and animals will not be recovered from this anaesthetic but will be humanely killed. After death organs may be removed from the animal and imaged to see if the labelled-drug is present and/or the organs can be used for further work/analysis.

Protocols 4-8 - Under general anaesthesia animals will have tumour cells implanted. This can be under the skin, into the bloodstream, into the abdomen or in a fat pad under the nipple. These animals will experience some discomfort after surgery and some mild to moderate pain which will be minimized with analgesics and animals will be very closely monitored. Tumour growth will then be typically measured three times per week using either an electronic measurement device (callipers) or by a non- invasive imaging machine. When tumours reach the size/burden needed to start treatment (around the size of a watermelon seed for a solid tumour), animals will be placed into groups based on the size/burden of their tumours so that all animals have tumours of similar size. Animals that will be dosed with the same drug will all be re-housed in groups together. Substances may be administered to the animals using standard routes (e.g. intravenous, subcutaneous, intraperitoneal, orally) and typically, animals will experience mild, transient pain but no lasting harm from administration. Provision of supporting tolerability data or acute phase tolerability studies means that the frequency of treatment- related adverse effects is uncommon in these studies. If the administration of substances is required for prolonged periods, a separate slow-release drug device may also be implanted under the skin of the animal. Animals may have blood, urine or faecal samples taken (urine and/or faeces obtained via non-invasive methods) and this will lead to mild and transient discomfort. At the end of the project the animals may have final samples taken under non-recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and may experience mild distress and no pain. In some studies where the tumours are sensitive to hormones to help the tumours grow, it may be necessary to supplement the animals with hormones using the most refined method (e.g. addition to the food and/or water, slow release implants under the animals skin). In other situations it may be necessary to perform studies where all of the influence of hormones are removed and for this we will use castrated animals from specific breeding establishments. At the end of the study, which typically will last no longer than 6-8 weeks, animals will be killed by a humane method.

Protocol 9 - In order to try and mimic the human immune system we may humanize animals by injecting adult human blood cells into mice, either by the bloodstream or into the abdomen. A small volume of blood (e.g. ~0.1ml) may be taken around 21 days post injection to confirm the human immune cells have multiplied within the animal. Substances may be administered to the animals using standard routes (e.g. intravenous, subcutaneous, intraperitoneal, orally) and typically, animals will experience mild, transient pain but no lasting harm from administration. Provision of supporting tolerability data or acute phase tolerability studies means that the frequency of treatment-related adverse effects is uncommon in these studies. If the administration of substances is required for prolonged periods, a separate slow-release drug device may also be implanted under the skin of the animal. Animals may have blood, urine or faecal samples taken (urine and/or faeces obtained via non- invasive methods) and this will lead to mild and transient discomfort. At the end of the project the animals may have final samples taken under non-recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and may experience mild distress and no pain. Animals will be monitored closely throughout the study for signs of rejection as graft versus host disease (GvHD) can develop. GvHD is expected to develop ~28 days after injection of the human blood cells into the animals. The signs of GvHD are well known and we have a specific scoring system for measuring the extent of GvHD. Animals are expected to show a combination of signs including weight loss, reduced activity, changes in posture, fur texture and skin integrity. Animals will be killed by a humane method when the signs of GvHD exceeds the

limit in our scoring system. These studies do not typically last more than 6 weeks from injection and animals should not experience and pain, distress or lasting harm.

Protocol 10 - ApcMin mice spontaneously develop 'cancer like' growths throughout the small and large bowel, bleeding from the growth/s is expected from ~18 weeks of age resulting in the development of anaemia as the disease progresses. In these studies, animals will be randomly assigned to treatment groups by age. All animals will be given either no dose, vehicle and/or test substances, either alone or in combinations. Typically, mice may be given test substances for up to eight weeks, via drinking water and/or one other route starting between 6-10 weeks of age. Animals may have blood, urine or faecal samples taken (urine and/or faeces obtained via non-invasive methods) and this will lead to mild and transient discomfort. At the end of the project the animals may have final samples taken under non- recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and may experience mild distress and no pain. At the end of the study, which typically will last no longer than 5 months (from birth of mice), animals will be killed by a humane method.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice may have surgery under anaesthesia to implant cancer cells at a specific location, or to implant a device under the skin that can release substances slowly. The mice are expected to recover quickly from the procedures and will be given painkillers and post-operative care. Animals will be pain scored for up to 3 days post-operative and any animals with a pain score of 1 or above on day 4 will be humanely killed.

Tumour growth will be monitored closely and if the tumour exceeds a pre-defined endpoint the animal will be humanely killed. If the tumour impairs the normal function of the animal (e.g. movement), the animal will be humanely killed. If any clinical signs are observed (e.g. hunched posture, subdued behaviour - responsive when provoked) the animal will be humanely killed if the animal does not return

to normal clinical condition within 2 hours. If the animal is in a poor clinical condition and/or un- responsive they will be immediately humanely killed.

Surgical procedures and/or administration of substances may cause body weight loss and if this body weight loss exceeds 15% the animals may be placed on a dosing break. Mice with body weight loss of ≥18% will be humanely killed. Mice with body weight loss ≥15% will be monitored for a maximum of 2 further consecutive days for improvement in body weight. If body weight does not recover to <15% during this time the animal will be humanely killed. Some substances can give rise to undesirable effects (e.g. vaccinia virus), if no improvement is seen in affected animals after 8 days they will be humanely killed. Repeated subcutaneous or topical administrations may cause skin abrasions, if these exceed 1.5 mm diameter, become wet or do not improve after 10 days monitoring, the animal will be humanely killed.

Some tumours may need hormones to grow (e.g. oestrogen-dependent breast/ovarian models). These hormones can be delivered as a supplement and is typically given by addition to food and/or water.

The hormones are usually given before the tumour cells are implanted. Where animals are given substances in diet and/or water, this is not expected to cause distress, but may sometimes result in body weight loss if the diet and/or water is unpalatable.

Supplementation with hormone can increase the development of sores around the genitals (urine scald) and bladder stones, so animals will be monitored frequently during body weighing. The presence of bladder stones will result in an enlarged bladder and a swollen abdomen. The animals are expected to experience some discomfort and some mild to moderate pain. Any animals displaying an enlarged and hardened bladder, visibly noticeable stones within the bladder, a swollen abdomen and/or deterioration in clinical condition will be killed by a humane method.

Tumour Growth and Development

ApcMin mice will spontaneously develop 'cancer like' growths throughout the small and large intestines with associated bleeding from ~18 weeks of age, ultimately resulting in anaemia. As the tumour(s) get bigger (i.e. number and/or size of growths) animals are expected to experience discomfort and mild or moderate pain. Animals will be checked frequently and assessed closely for clinical signs including pale ears, paws, bloody discharge from the anus, body weight loss, disturbed normal behaviours and other signs indicating loss of body condition.

Tumours placed under the skin or in the fat under the nipple, can sometimes ulcerate. Any ulceration is monitored frequently during measurement and given a grade from 1-4; grades 1 & 2 are closely monitored and animals with grades 3 & 4 are killed by a humane method.

Tumour growing within the abdomen may also attach and grow on other internal organs. They may also develop fluid in the abdomen which leads to swelling and maybe some discolouration of the abdomen. If this is seen the animal will be humanely killed.

Tumours that have been injected into the bloodstream can, depending on the cell line used:

- Start growing in the lungs (e.g. lung metastasis models) which may result in changes in breathing, and/or pale ears and paws. If this is observed the animal will be humanely killed.

or

- Start growing in several tissues (e.g. spleen, bone marrow, fore and hind-limbs, spine). Animals may show problems with mobility and may also have difficulty using one or more of their limbs. If this is observed or there is evidence of any discomfort/pain, the animal will be humanely killed.

Humanization

Animals may be injected, either into the bloodstream or the abdomen, with human blood cells and are expected to experience mild, transient pain from the injection. Approximately one month after injection animals will typically show signs of acute rejection which will be monitored closely using a scoring system.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Protocol number / Title	Expected Severity	Species / %age of animals showing severities
Non-tumour bearing protocol - blood/tissue sample collection	Non-recovery	Mouse / 100% Non-recovery
Non-tumour bearing - tolerability	Moderate	Mouse / ~20% Moderate, ~80% Mild
PK/Biodistribution studies	Moderate	Mouse / ~20% Moderate, ~80% Mild
Subcutaneous tumour models	Moderate	Mouse / ~20% Moderate, ~80% Mild
Experimental metastasis models - intraperitoneal ascites	Moderate	Mouse / ~50% Moderate, ~50% Mild
Experimental lung metastasis	Moderate	Mouse / ~90% Moderate, ~10% Mild
Haematopoietic tumour models	Moderate	Mouse / ~60% Moderate, ~40% Mild
Mammary fat pad tumour models	Moderate	Mouse / ~30% Moderate, ~70% Mild
Mouse humanization	Moderate	Mouse / ~80% Moderate, ~20% Mild
Efficacy of candidate anti-cancer agents in Genetically Engineered Mouse Models (GEMM's) of colorectal cancer	Moderate	Mouse / ~90% Moderate, ~10% Mild

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In vitro / in silico methods may be useful to predict absorption, distribution, metabolism and elimination of new anti-cancer drugs properties, as well as target engagement and pharmacology assessment of a well-defined compound series. This can be used as an initial screen to help to prioritize compounds for further evaluation in animals, however in vitro / in silico methods are unable to predict accurately the full in vivo effects that a novel compound may have when administered to animals in terms of any potential off target effects or toxicity. The tumour/host interaction is complex with many different cell types and systems (e.g. the blood and lymphatic systems) interacting and communicating with each other. Currently there are no invitro systems that are able to replicate these complex processes.

Which non-animal alternatives did you consider for use in this project?

In vitro methodologies have replaced animal use to a degree in cancer research, particularly in the development of screening assays to refine compound selection, target identification, off-target toxicity or toxicity versus normal tissue cell lines. These in vitro assays can certainly be used to guide and refine the steps prior to moving into in vivo studies, and thus minimise subsequent animal use. Our company is actively working in the field of in vitro assays and we are developing cutting edge technologies to enhance the in vitro studies we offer, which recently include the development of 3D tissue assays (called organoids), offering customized cell screening assays of up to 250 cell lines and the use of novel imaging methods to allow precise drug/tumour interactions to be observed.

Why were they not suitable?

Although in vitro tumour growth assays are routine within our company, and can help in the identification of suitable cell lines or lead candidates, there is still a need to use animals for the project as the 2D and 3D in vitro assays still do not optimally mimic what happens in vivo, therefore in vivo models are still required prior to progression of new drugs into human clinical trials.

Research and development of anti-cancer agents involves a multi-step process in which in vivo studies form an important part of the regulatory process linked to the approval of new drugs. Drug products not previously authorized for human use must undergo a submission application process before progressing to human trials, and applicants are expected to support that process with in vivo pharmacology as part of the submission.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 have estimated to use is based on several factors including:

- the client demand for the model and how this has changed from previous years
- assessing the change in focus based on published articles
- where research council funding drives are directed, to ensure that we can meet demand for specific models in the relevant areas

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The use of in vitro studies can be used to identify lead compounds, evaluate dose ranges confirming target modulation/expression and relative off-target toxicity which can be used to inform on relevant doses and exposure (e.g pharmacokinetics) for use in pharmacology based studies and pilot toxicity studies.

Study format will vary on a per project basis, and typically range from 2-20 groups; test agents may be blinded dependent on the particular project requirements. Studies will include at least one control arm, and control arms for both route and frequency, as well as changes in vehicle composition, will be employed where relevant. Use of positive control groups (either a standard of care or an agent that induces significant growth inhibition) will be regularly employed to ensure the model is behaving consistently. For combination dosing, dosing should be balanced across all study groups.

Group numbers will be based on power analysis (supported by our dedicated statisticians in China) but typically range from 3-20 mice/group across all protocols in this license. For efficacy testing of internal bioluminescence, variability tends to be higher due to absorption and reflection of bioluminescent signal by internal organs and tissues, therefore higher group number are employed to maintain study power.

The model development stage of this project will be used to determine statistical power to ensure the minimum number of mice are used in a study design, but with the ability to still achieve robust scientific endpoints. The use of imaging technologies can also reduce the number of animals required to generate study outcomes as model variation can be improved by eliminating mice which do not develop the disease appropriately.

Guidance on experimental designs and methods of analysis of the results will be obtained from NC3Rs (https://www.nc3rs.org.uk/experimental-design) and by attendance at relevant

online seminars that are being hosted by NC3R to ensure that we are keeping up to date with the most relevant design guidelines and data analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The use of short-term tolerability assessments may be avoided if supporting data is already available in a relevant model (the correct animal strain, tumour model, compound batch and dosing route/regimen).

Pilot studies will be performed on any newly developed animal model, part of which will facilitate the identification of experimental variation and this will be used directly in powering studies to achieve statistical significance. The use of a structured committee approach to the decision on which models progress to the development stage, ensures that the process is both reflective of the scientific aims and conducted in a way that minimizes the use of animals to achieve the project aims. Study design will incorporate numerous controls both in statistical design, and the application of correct vehicle and dosing controls groups. Each study is governed by an experimental protocol and the generation, review and application of protocols in delivery of studies is governed by SOPs and underpinned by the QMS system. Protocols include a statement of the objective(s); a description of the experiment, covering such matters as the experimental treatments, the size of the experiment (number of groups, number of animals/group), and the experimental material; and an outline of the method of analysis.

Where possible, we will make appropriate arrangements to randomly assign animals to experimental groups and blind studies. Experiments will be planned, where possible, so they can be published in accordance with the NC3Rs' ARRIVE guidelines.

For efficacy study formats, candidate anti-cancer agents can typically be challenged in one of two scenarios:

1. Efficacy against established tumours: Tumour-bearing mice are randomised to control and treatment groups based on either bioluminescent signal or tumour volume measurement at the earliest time point that will deliver the scientific objective. For slow growing models a static phase may precede a growth phase which is progressive, and typically for these models dosing will start when the progressive growth phase has been identified.

2. Tumour prevention: In these studies mice are randomised to control, and treatment groups based on body weight prior to tumour inoculation; the group numbers will typically be larger as there is no provision for removing outliers or animals without established tumours prior to commencement of treatment. Dosing will typically commence from up to 0-14 days prior to tumour inoculation and continue thereafter, with the pre-dosing duration driven by the agent/strategy under investigation e.g. administration of a blocking antibody may only be required on the day of implantation, whereas a bacteria preparation designed to increase immune response may require 14 days for complete colonization.

For studies looking at efficacy (Objective 2) with already established models, implantation of an excess of mice helps reduce overall total animal use on study by minimizing study variation at randomisation by allowing the statistical removal of outliers (e.g. Stem Leaf

analysis), inconsistent or non-growers, and animals displaying early clinical signs that may compromise study design and/or animal welfare e.g. early signs of tumour disseminating in non-target organs.

Where relevant and available, the use of longitudinal imaging modalities, either bioluminescent and/or fluorescent, will be used to reduce the numbers of animals used. For example, for an orthotopic model whose internal tumour dimensions cannot be measured, tumour growth would normally have to be characterized by timed terminations of multiple study groups. Using imaging technologies, this can be assessed in a single cohort of animals by utilizing multiple image points, longitudinally on the same cohort of study animals. Frequency of imaging sessions will be balanced with model duration to ensure that the growth is adequately assessed whilst minimizing the number of procedures carried out. The use of imaging technologies can also reduce the number of animals required to generate study outcomes as model variation can be improved by eliminating mice which do not develop the disease appropriately (e.g. the tumour fails to seed in the expected location) or refining the model so this is minimized.

Our company offers a six subcutaneous tumour models quarterly screen, whereby multiple clients can subscribe. This means that control animals (e.g. vehicle, SoC) can be shared across clients thus reducing total number of animals used compared to carrying out individual studies.

Too strict limits for removing an animal from study due to tumour ulceration in mouse models may result in early termination of experimental mice when the degree of ulceration may actually have very little negative impact on animal welfare. This would prematurely reduce group size and thus possible affect the scientific outcome. To overcome this then you could look to increase the number of mice on study so that the study size remains adequate to answer the question asked, however this would lead to an increase in overall animal usage, which we want to avoid. An alternative is to use a more detailed ulceration score (with animal welfare central) that would allow more mice to remain on study resulting in the overall reduction of use of mice in these models with no compromise on animal welfare.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 the generation of tumours from tumour cell lines grown in vitro, (wild-type, labelled or modified genetically), cells undergo a highly refined process (driven by SOPs, underpinned by the Quality Management Systems) with numerous checkpoints and controls, to ensure only high quality viable aseptic cells are used for implantation into mice.

In preclinical studies in SCID mice treatment with vaccinia virus results in the formation of pox-lesions (red papules) on the tail which is a typical symptoms of systemic infection by vaccinia virus 4-5 days post intravenous injection which improve 8-10 days later. Lesions will be a dry raised red papule, but if lesions appear wet or show signs of infection then the animal will be humanely killed. If the lesions do not show signs of improvement after 8 days from first appearance they will be humanely killed. In addition the injection of the virus will be carried out towards the base to reduce the length of the tail exposed to the virus and reduce the frequency of the lesions. Pox-lesions may also appear on the feet and nose of the animal, animals will be humanely killed if these appear.

Generally the use of non-invasive longitudinal bioluminescent imaging and/or ultrasound guided measurements will be used to refine the methods used for all orthotopic cell line models. As well as minimizing animal suffering, it allows the opportunity for the determination of a statistically significant result ahead of a scheduled termination, thus potentially reducing the duration and use of regulated procedures on animals. Any animal welfare concerns can also be investigated using ultrasound, assisting in determining causes of symptoms in a non-invasive manner.

Supporting tolerability data or acute phase tolerability studies will minimise the frequency of treatment dosing related adverse effects in later stage studies performed under protocols 4, 5, 6, 7, 8 & 10 however, body weight will be monitored daily during the dosing phases and will be used to guide intervention with persistent adverse clinical signs. Animals showing subdued behaviour patterns even when provoked, will be killed by a humane method regardless of body weight measures.

Orthotopic models (protocols 5, 6, 7 & 8) are known to better model cancer in patients as tumour grows in the correct environment which facilitates spread to other organs as seen in patients and show a reduced response to chemotherapy therefore providing more relevant information on the drug/s. The use of imaging is also a refinement as data from the internal tumours can be captured in real time, providing additional data that wouldn’t normally be visible, only using animals that show tumour, and minimize animal suffering, as it allows the opportunity for the determination of a statistically significant result ahead of a scheduled killing of the mice, thus reducing the duration of model and regulated procedures.

Animals used in protocols 4, 5, 6, 7 & 8 may have tumours implanted:

- on the side of their body under the skin
- into the abdomen
- into the bloodstream
- into the mammary tissue under a nipple

Animals used in protocol 9 will have human peripheral blood mononuclear cells (PBMC's) injected into either the bloodstream or the abdomen.

ApcMin mice used in protocol 10 will spontaneously develop multiple polyps/adenomas and are used as a model for human colon cancer.

Some of these models have a better relevance to patients, but are also technically more complex and may require imaging (either bioluminescent and/or ultrasound) to track the growth of the tumour. For bioluminescent imaging, this is achieved by using tumour cell lines for implantation that are altered to emit light, which can then be captured by an imaging system specifically designed for small animals. For monitoring of tumour growth by ultrasound, there is no requirement for the cell line to be altered to emit light prior to implantation as the ultrasound is capable of detecting the presence of the tumour mass anywhere in the body. The exception to this is when human patient tumours are implanted which are difficult to bioluminescent/fluorescent tag and therefore monitoring of tumour burden in these models is via the use of daily monitoring for the appearance of well-known and documented clinical signs and humane endpoints. Each model has its own clinical signs and defined humane endpoints, which where appropriate are described in the individual protocols for each model.

For non-tumour bearing protocol - blood/tissue sample collection (protocol 1), all animals will be anesthetised prior to blood and/or tissue samples being taken so they cannot feel anything, they will not be allowed to wake up and be killed immediately after sampling.

When undertaking tolerability studies, the rodent strain/s selected will typically be consistent with the strain proposed for the follow-on efficacy study. However, where it is considered unlikely to impact on the scientific outcome of the study, the background strain of a mutant (immunocompromised) or genetically altered (GA) mouse strain may be employed to minimise the use of these types of rodents.

Non-tumour bearing tolerability studies (protocol 2) are the first time that dose/s of potential new anti-cancer agents will been given to animals. As it is difficult to predict possible effects and the nature of anti-cancer agents or interactions it's likely that some animals will experience body weight loss which will be limited at up to 18%. Clinical effect may also be seen immediately post treatment such as transient disturbed normal behaviour repertoires (e.g. hunched posture, subdued behaviour patterns - responsive when provoked, stretching, belly pressing) and will be monitored closely. By dosing small numbers of animals first to find doses that are tolerated, suffering is avoided in larger numbers of animals in later stage efficacy studies.

Tolerability testing of single or combination treatment/s will typically be carried out at the highest proposed dose level. If the initial dosing regimen produces evident toxicity, the study will be stopped; the dose will be reduced by a stepped approach (~30-50%) and tested in a new group of animals. For studies looking to generate maximum tolerated dose (MTD) of single or combination treatment/s, the doses will be increased by a stepped approach (~30-50%) once the preceding dose level has been confirmed to be tolerated (e.g. stable/increasing body weight and normal clinical signs).

For PK/Biodistribution studies (protocol 3) it may be the first time that dose/s of potential new anti- cancer agents will been given to animals. As it is difficult to predict possible effects and the nature of anti-cancer agents or interactions it's likely that some animals will experience body weight loss which will be limited at up to 18%. Clinical effects may also be seen immediately post treatment such as transient disturbed normal behaviour repertoires (e.g. hunched posture, subdued behaviour patterns - responsive when provoked, stretching, belly pressing) and will be monitored closely.

Typical rodent experiments use a single dose with 4–8 time points (3 mice per point) over 1–72h for small molecules as they usually have a relatively quick clearance rate, and time points over 1–28 days for biopharmaceuticals due to their slower clearance rate. Animals may be sampled in-life or under general anaesthesia (e.g. blood/tissue sample, image etc.).

The endpoint for PK studies is the defined sampling schedule which is selected based on known compound properties or the attributes of similar compounds. The relatively short dosing duration means that the prevalence of treatment-related adverse effects is uncommon in these studies, however, where there are adverse clinical signs documented e.g. subdued behaviour patterns even when provoked animals will be killed by a humane method.

For subcutaneous tumour models (protocol 4) tumour engraftment will take place by injecting tumour cells under the skin, but may also be carried out by making a small cut in and placing tumour tissue under the skin, it typically takes 1-4 weeks for tumours to grow. The tumours are surface presenting so development and growth can be assessed by regular calliper measurement (length and width measurement). For hormone dependent models (some oestrogen-dependent breast/ovarian models, and some androgen-dependent prostate models) hormone supplementation using the most refined method that results in consistent tumour growth will typically commence up to 2 weeks prior to implantation. Experiments will normally consist of a continuous dosing regimen of 2-4 weeks, with a typical study duration ranging from 4-8 weeks. The primary humane endpoint for tumour size is a mean diameter ≤15mm for model development and efficacy testing studies or ≤12mm for donor mice. Mice bearing tumours that are close to this limit that are likely to reach or exceed this size by the next measurement, should be killed by a humane method prior to that point. With some tumour cell lines, we may see ulceration on the tumours as they grow (typically those that grow faster). The use of an appropriate scoring system with defined endpoints and escalated actions is key to the refinement of this process, and ensures that the welfare of the animals isn’t compromised and the risk of harm is minimised throughout the model duration. Some tumour cell lines can grow invasively into/through the body wall and/or into underlying musculature, ultrasound monitoring for this will be performed, where appropriate, to minimise the impact to animal welfare.

For **experimental metastasis models - intraperitoneal ascites (protocol 5)** tumour engraftment is established by a cell injection directly into the abdomen and depending on the specific cell line model under challenge, it typically takes ~1-2 weeks to establish tumours. Pilot studies (Objective 1) will be used to further refine the model (e.g. cell injection volume, concentration). For hormone dependent models (some oestrogen-dependent breast/ovarian models, and some androgen-dependent prostate models) hormone supplementation using the most refined method that results in consistent tumour growth will typically commence up to 2 weeks prior to cell injection. Experiments will normally utilise a continuous dosing regimen of 2-4 weeks, with typically study duration ranging from 4-8 weeks. The duration to reach a defined tumour size (TABL), may also be used as a scientific endpoint (surrogate survival). The primary humane endpoint for all cell line models used in this experimental ascites model, is progression of ascites (tumour cells in ascitic fluid) and diffuse peritoneal tumour (small tumour masses loosely adhered to internal organs/structures) resulting in abdominal distention and discolouration. For each cell line model an intervention measure of TABL and/or daily monitoring for the appearance of well-known and documented clinical signs mentioned above (unlabelled

cells e.g PDX material) will be determined during pilot studies, prior to the onset of any adverse effects. (e.g. ≤1x109 photons/second for A2780 (whole body measurement) and the intervention measure of TABL will be constantly revised on the basis of ongoing study review). Some tumour cell lines can grow invasively into/through the body wall and/or into underlying musculature, ultrasound monitoring for this will be performed, where appropriate, to minimise the impact to animal welfare.Furthermore, if tumours grow at off-target sites, animals will be killed by a humane method.

For **experimental lung metastasis (protocol 6)** tumour engraftment is established by a cell injection directly into the bloodstream and depending on the specific cell line model under challenge, it typically takes ~1-2 weeks to establish tumours. Pilot studies (Objective 1) will be used to further refine the model (e.g. cell injection concentration). For hormone dependent models (some oestrogen-dependent breast/ovarian models, and some androgen-dependent prostate models) hormone supplementation using the most refined method that results in consistent tumour growth will typically commence up to 2 weeks prior to cell injection. Experiments will normally utilise a continuous dosing regimen of 2-4 weeks, with typically study duration ranging from 4-8 weeks. The duration to reach a defined tumour size (TABL), may also be used as a scientific endpoint (surrogate survival). The primary humane endpoint for all cell line models used in this experimental lung metastasis, is changes in respiration (rate and depth), pale extremities or changes in behaviour that would indicate an increased respiratory burden. For each cell line model an intervention measure of TABL and/or daily monitoring for the appearance of well-known and documented clinical signs mentioned above (unlabelled cells e.g. PDX material) will be determined during pilot studies, prior to the onset of any adverse effects. (e.g. ≤5x109 B16F10 or ≤1x1010A431 photons/second (whole body measurement) and the intervention measure of TABL will be constantly revised on the basis of ongoing study review). Furthermore, if tumours grow at off-target sites, animals will be killed by a humane method.

Experimental metastasis models mimic later stages of disease progression (e.g. establishment at the metastatic site) that may be difficult to model using spontaneous metastasis models where primary tumour size may drive the model endpoint. Experimental metastasis models are therefore useful for assessing candidate anti-cancer agents directly targeting the development of metastasis, or metastatic treatment strategies which often differ to those used for primary disease in patients.

For **Haematopoietic tumour models (protocol 7)** tumour engraftment is established by a cell injection directly into the bloodstream to mimic human disease, depending on the specific cell line model under challenge, it typically takes ~1-2 weeks to establish tumours. Pilot studies (Objective 1) will be used to further refine the model (e.g. cell injection concentration). For hormone dependent models (some oestrogen-dependent breast/ovarian models, and some androgen-dependent prostate models) hormone supplementation using the most refined method that results in consistent tumour growth will typically commence up to 2 weeks prior to cell injection. Experiments will normally utilise a continuous dosing regimen of 2-4 weeks, with typically study duration ranging from 4-8 weeks. The duration to reach a defined tumour size (TABL), may also be used as a scientific endpoint (surrogate survival). The primary humane endpoints for systemic haematopoietic models is primarily metastasis to haematopoietic tissues which ultimately results in changes in normal behaviour, ataxia, splayed limbs, leading to partial or full paralysis of the limbs. For each cell line model an intervention measure of TABL and/or daily monitoring for the appearance of well-known and documented clinical signs

mentioned above (unlabelled cells e.g. PDX material) will be determined during pilot studies, prior to the onset of any adverse effects. (e.g. ≤1x1010 MV4-11 photons/second (whole body measurement) and the intervention measure of TABL will be constantly revised on the basis of ongoing study review).
Furthermore, if tumours grow at off-target sites, animals will be killed by a humane method.

For **Mammary fat pad tumour models (protocol 8)** tumour engraftment will take place by injecting tumour cells into the mammary tissue under a nipple, it typically takes 1-2 weeks for tumours to grow. The tumours are surface presenting so development and growth can be assessed by regular calliper measurement (length and width measurement) as well as BLI. For hormone dependent models (some oestrogen-dependent breast/ovarian models, and some androgen-dependent prostate models) hormone supplementation using the most refined method that results in consistent tumour growth will typically commence up to 2 weeks prior to implantation. Experiments will normally consist of a continuous dosing regimen of 2-4 weeks, with a typical study duration ranging from 4-8 weeks. The primary humane endpoint for tumour size is a mean diameter ≤12mm, mice bearing tumours that are close to this limit that are likely to reach or exceed this size by the next measurement, should be killed by a humane method prior to that point. With some tumour cell lines, we may see ulceration on the tumours as they grow (typically those that grow faster). The use of an appropriate scoring system with defined endpoints and escalated actions is key to the refinement of this process, and ensures that the welfare of the animals isn't compromised and the risk of harm is minimised throughout the model duration. Some tumour cell lines can grow invasively into/through the body wall and/or into underlying musculature, ultrasound monitoring for this will be performed, where appropriate, to minimise the impact to animal welfare. Spread of tumour to draining lymph nodes, lungs, liver, fore/rear-limbs and brain have been observed but the primary tumour masks the signal so BLI is only typically used to either monitor the spread using shielding protocols alongside tumour reading or measure signal in tissue/s that the tumour has spread to after the animal is killed by a humane method.

For mouse humanization (protocol 9) pilot studies (Objective 1) are undertaken to refine cell injection (e.g. injection site, cell concentration, volume injected). Human PBMC's are injected either directly into the bloodstream or into the abdomen, it typically takes ~3-4 weeks for the human patient cells to engraft which will be confirmed by blood sample analysis. Typical study duration ranges from 4- 5 weeks. The primary humane endpoint is the animals showing signs of graft v's host disease (GvHD) which results from attack of the animals immune system by the patient cells. The use of an appropriate scoring system with defined endpoints and escalated actions is key to the refinement of this process, and ensures that the welfare of the animals isn't compromised and the risk of harm is minimised throughout the model duration.

For **efficacy of candidate anti-cancer agents in GEMMs of colorectal cancer (protocol 10)** all animals will spontaneously develop colon cancer, this involves the growth of multiple polyps/adenomas throughout the small and large intestines. From ~18 weeks of age some animals will show associated bleeding from the anus resulting in anaemia. Experiments will typically consist of a continuous dosing regimen of up to 8 weeks, with overall study duration ranging from 9-10 weeks from animal arrival. As the tumours grow inside the animals and therefore currently not accessible for measurement/counting the primary humane endpoint is the development of anaemia indicated by the animals showing clinical signs of pale extremities, bloody discharge from the anus, body weight

loss and other clinical signs indicating loss of body condition. The use of an appropriate scoring system with defined endpoints and escalated actions is key to the refinement of this process, and ensures that the welfare of the animals isn't compromised and the risk of harm is minimised throughout the model duration.

Why can't you use animals that are less sentient?

Mice are the lowest species in which the knock out of the immune system allows growth of human tumours. Mice with a fully functioning immune system also provide the opportunity to investigate the immune system interplay with a mouse tumour.

The architecture of mammals is required to accurately model tumour development and spread to relevant organs. Work on human tissue and use of mixed tumour-derived cell types, especially when freshly derived from a patient should allow the most refined assessment of novel anti-cancer agents. Transgenic technology and can also be used to assess the importance of potential oncogenes and mice are the lowest species in which this technology can be applied to as this requires an appropriate mammalian architecture.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The development of relevant pre-clinical models of oncology in Objective 1 is a key stage for the evaluation of candidate anti-cancer agents to ensure the right models are being used to answer the questions being addressed in Objective 2. The following will be undertaken to minimize animal suffering across all models;

- Pilot studies for the establishment of new tumour cell lines and refinements to surgical techniques will be carried out on an ongoing basis and the advice of the vet (NVS) and/or the named animal care and welfare officer (NACWO) will be sought.

- Use of pilot tolerability studies to ensure there are no unexpected adverse effects associated with new models and/or unexpected toxicity because of tumour:drug interactions, and to ensure the drug levels used are not associated with any cumulative effects.

- All surgical procedures will be conducted in line with established welfare guidelines on aseptic surgery, using suitable anaesthesia along with peri and post-operative analgesia. Where there is a scientifically justifiable reason for concern that the mechanism of action of an analgesic regimen may affect cell growth and tumour initiation, advice will be sought from the NVS as to suitable alternative forms of analgesia i.e. those with a different mechanism of action.

- All studies will be conducted in accordance with established welfare guidelines. Presentation of adverse clinical signs, behaviour patterns or body weight loss relating to treatment or model progression should be de-risked by supporting work summarised above, and managed as detailed in the relevant project plan and protocol sections that will accompany each individual study.

- Any in-life sampling or terminal sampling will be in line with established welfare guidelines and micro-sampling regimens will be utilised where study design supports this.

- The frequency of dosing will be such that animals fully recover between dosing occasion and will not suffer more than transient pain and distress and no lasting harm and there will be no cumulative effect from repeated dosing.

- For models that have a defined organ of tumour establishment (e.g. lungs), evidence of tumours seeding within other organs/sites, without evidence of a primary tumour within the intended organ, will result in animals being humanely killed.

- Animal husbandry and enrichment will be constantly reviewed and updated according to best practices. Cardboard tubes and housing will be used where possible to aid with wound healing following any surgical intervention.

- Animals that are prone to hind limb issues, e.g mice bred on a SCID background, will be given soft bedding and non-adverse handled. Food may also be placed directly into the cage to prevent animals having to reach up onto their hind limbs in order to access food hoppers.

- The use of supplemented diet or drinking water may be used for both candidate anti-cancer agents as well as hormone supplementation, but in such circumstances, care should be taken to carefully monitor intake to ensure that that the change in composition doesn't affect normal feeding/drinking behaviour. For hormone dependent models (some oestrogen-dependent breast/ovarian models) hormone supplementation using the most refined method that results in consistent tumour growth for efficacy testing will be utilised (namely supplementation via the drinking water, hormone rods, pellets, or mini-pump devices). Alternative supplementation in the diet and/or water has been tested and further refinements will be evaluated.

- If the animals have oestrogen supplementation then there may be evidence of the following: Bladder calculi may lead to urinary retention resulting in an enlarged bladder with abdominal distension. We may use ultrasound to monitor the bladder calculi where appropriate. Animals may also experience urine scald. Any animal exhibiting these signs, or evidence of a grade 3 urine scald (reddened, broken skin with possible discolouration and wet around the genital area), or a deterioration in clinical condition will be humanely killed. Oestrogen supplementation via drinking water has, in our hands, resulted in growth of an oestrogen-sensitive cell line and urine scald and bladder calculi were not observed. Where possible we will look to validate our models using oestrogen supplementation via the drinking water, however this may not be possible for cell lines that need high oestrogen concentrations. We have also found that using certain strains of mice e.g. NSG avoids the occurrence of bladder calculi and urine scald following oestrogen supplementation, and where possible, this strain of mouse will be used in new model development or re-validation studies.

- Where a statistically significant effect on study goals can be determined prior to the end of the scheduled dosing phase, the study will be terminated at that point.

- Through continual professional development, our company seeks to improve and implement new techniques for current/new models, which are developed through dialogue and guidance from the NVS or alternative veterinary expertise, to ensure that the most refined methods are considered.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Surgical procedures will be carried out in accordance with the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery.

For administering substances the Laboratory Animal Science Association Good Practice Guidelines for administration of substances will be followed.

Frequency and volume of blood collection will be carried out in accordance with the following guidance https://www.nc3rs.org.uk/3rs-resources/blood-sampling)

Planning and reporting of experiments will be in accordance with Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) and Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The following practices are already in place:

- Regular interaction with the National Centre for the Replacement, Refinement and Reduction in Animals in Research website (https://www.nc3rs.org.uk) and subscription to regular newsletters.

- Regular discussions with named role holders (i.e NIO, NVS, NACWO & NTCO) and In-vivo colleagues (i.e animal scientists, technicians and care staff).

- Attending appropriate training courses and conferences or seeking feedback from colleagues attending these events.

- Liaising with AWERB members.

During AWERB meetings, internal R&D data is presented and discussions around future R&D takes place. During this meeting advice is openly given on latest and best practices to include on the R&D studies. Following completion of the model development phase, a report will be generated and the outcomes of the model development process will be carefully reviewed by the AWERB/R&D committee before the model is considered suitable for use in client studies. As part of the ongoing commitment to the highest levels of scientific and welfare, a regular review period will be set up for each model following completion of the model development process. This will look to follow-up on the current use and applications of the model to ensure that the most refined science and animal welfare is being utilised. Where areas of potential refinement are identified, these will be assessed as part of further pilot studies.

44. Single and multi-modality cancer therapies 2023

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Therapy, Ultrasound, Combination therapy, Therapy enhancement

Animal types	Life stages
Mice	adult, juvenile
Rats	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To develop novel combinations of physical therapies (such as ultrasound therapy and radio-therapy) and "systemic" therapies (i.e. medicinal substances injected into the blood stream (e.g. chemo-therapy, viruses that kill cancer, or molecules that heighten the body's natural defences (its immune response)) either alone or in combination to treat cancer more effectively and with fewer or less severe side effects. For example, therapy ultrasound exposure of a tumour can increase the uptake of drugs into the tumour allowing more effective treatment using lower drug doses which reduces the risk and severity of 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?

Cancer now affects more than 1 in 3 people, and as people live longer, the rate of cancer diagnoses will continue to increase. This licence aims to improve cancer therapy in a variety of ways.

Cancer treatment can have a curative aim i.e. one that results in disease-free survival or a palliative aim i.e. one that reduces cancer-related symptoms in order to improve quality of life.

Ultrasound therapy offers a non-invasive, bloodless technique for treating cancer using devices located outside the body. It can be used to heat tissue or to mechanically shake tissue enough to liquify it. It can also be used to block the blood supply to tumours, starving them of essential nutrients. Since ultrasound therapy has very limited effect on normal tissue, treatments can be repeated multiple times.

In addition, there are more traditional methods of treating cancer, such as chemo-therapy (using drugs delivered to the blood stream) and radio-therapy (in which high energy electrons damage the fundamental building blocks of cells, their DNA).

Broadly, there are physical therapies such as ultrasound and radio-therapy which are targeted at cancer because they only produce a local effect, and "systemic" therapies (i.e. ones where the treatment is delivered to the whole body rather than a local target) such as chemo-therapy, each of which has strengths and weaknesses. Combining different therapies offers the potential to enhance their effectiveness and/or reduce their side effects.

For example, cells with low levels of oxygen, such as the centre of tumours are less responsive to radio-therapy. But heating tumours with ultrasound before or after radio-therapy can enhanced the cell killing effect. This in turn allows the use of lower levels of radio-therapy which reduces the damage to normal tissues, which do not become sensitised by heat to the extent that cancer cells normally do.

A second example is that some drugs have been shown to be very effective in killing brain cancer cells in the laboratory, but studies in animals have shown a lack of effectiveness due to the so-called blood- brain-barrier which lines the blood vessels in the brain preventing molecules from crossing into the brain tissue. Therapeutic ultrasound can be used with microbubbles injected into the blood stream to safely and temporarily open the blood brain barrier to allow drugs into brain tumours.

These are two examples of combination (i.e. multi-modality) therapy, but there are a vast array of other options including combination with immune-therapy (in which the hosts ability to fight invading cells is stimulated) or oncolytic viruses (where viruses have a natural tendency to infect and kill tumour cells). We are interested in exploring the most promising options within a hugely diverse range of possible combinations.

We tend to concentrate on the highest unmet clinical need, for example where currently life expectancy may be 12 months or less. We would also like to produce smarter, kinder treatments i.e. more effective (to prolong life) with less severe side effects (to provide a better quality of life).

What outputs do you think you will see at the end of this project?

Expected outputs are:

the identification and better understanding of promising new cancer therapeutic strategies that will be shared via international conferences and peer-reviewed journals and

continuing to design and build our own clinical therapy systems and/or use commercially available clinical systems for cancer clinical trials based on these pre-clinical investigations.

Who or what will benefit from these outputs, and how?

In the short term, these studies should result in conference presentations within 6 months of completing data analysis and peer-reviewed publications around 12 months after study completion. These will initially benefit other academics/clinicians working in the same areas.

In the medium term, clinical translation (i.e. performing new treatments on humans in clinical trials), benefiting clinicians, generally takes another 5-10 years of effort.

In the longer term, only following successful clinical trials, will patients begin to benefit and there be the potential for commercial exploitation (i.e. the development and manufacture of medical products) benefiting the economy and wider public.

How will you look to maximise the outputs of this work?

Sharing at international conferences and in peer reviewed journals will maximise academic access to the outputs.

Expanding our collaborative network which spans academic and clinical institutions within the UK, and across Europe.

Species and numbers of animals expected to be used

- Mice: 3900
- Rats: 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.

Adult and juvenile rodents (mice and rats) are the smallest mammals with the lowest capacity for emotional feelings in which we can grow tumours like those that affect people, in which to test the anti- cancer treatments we are developing. Typically, animals younger than 10 weeks at the beginning of the study will be used. For studies where an intact immune system is important, animals between 10-25 weeks old are preferred.
Typically, what will be done to an animal used in your project? Tumour implant:
Most animals (>90%) will have a tumour implanted (by injection of cells) either under the skin or within an internal organ to mimic human cancer. Internal organ tumours will

normally require minor surgery, generally an approximately 1cm skin incision. Because physical therapy requires tumours under the skin to be implanted in a specific location, this is normally performed under general anaesthesia lasting from ~5 to ~20 minutes to avoid the animal moving and experiencing pain or distress. Recovery from anaesthesia is very quick (minutes) and pain relief is given to overcome surgical pain.

A minority of animals (up to 10%) will be used to understand the effect of therapies on normal tissue rather than tumours. This is important because human treatment routinely involves treating a "margin" of normal tissue around a tumour to ensure that all the tumour cells are targeted.

Therefore, the effect of treatment on normal tissue is important in terms of treatment safety.

Imaging: typically, imaging is used to monitor tumour growth pre-therapy and tumour response post- therapy, sessions under anaesthesia generally last from 10 to 60 mins. A minority of tumours may be imaged without delivering therapy either as controls for therapy or imaging studies. Often substances will be injected intravenously that provide the imaging information (e.g. radio-isotopes) or improve contrast (contrast agents).

Blood sampling: serial sampling may be used to monitor response to therapy (including obtaining pre- treatment, baseline data).

Therapy: Tumours (or sometimes normal tissue) will be exposed to a single therapy or a combination of therapies. Therapies may be loosely classified as physical or systemic as explained below.

Physical therapies such as ultrasound therapy and radio-therapy are usually delivered from outside the body without surgery but under general anaesthesia. Therapy sessions usually last less than 1 hour, but with pre- and/or post-therapy imaging, anaesthesia could be needed for up to 3 hours. Therapy may consist of a single session, or sessions that are repeated e.g. weekly or, in the case of radio- therapy, daily (5 days on and 2 days off), and may continue for several weeks up to a few months.

Before, during or after physical therapy, substances may be administered that:
enhance the therapeutic effects of a physical therapy (e.g. microbubbles for ultrasound therapy or radio-sensitisers for radio-therapy have their delivery into tissue (tumours) and/or their medicinal effect enhanced by the physical therapy

Systemic therapies such as chemo-therapy, gene-therapy, viral-therapy, antibody-therapy, immune- therapy, etc., are usually delivered without anaesthesia via injection or oral dosing, daily or less frequently, with dosing continuing for several weeks up to months.

Physical and systemic therapies can be combined in many and varied ways. Examples include:

1.using ultrasound to open the blood-brain-barrier after the injection of a mixture of chemotherapeutic drug and ultrasound therapy enhancing microbubbles to deliver drug to tissue it cannot normally penetrate (simultaneous physical/systemic)

2.mildly heating tumours with ultrasound hyperthermia before (or after) radio-therapy treatment to enhance therapy by making cells more sensitive to radiation so that lower doses can be used (physical followed by physical)

3. stimulating an anticancer immune response using ultrasound exposure in combination with a course of drugs that stop the immune response from being turned off (physical followed by systemic)

Each of the above single or combined therapies may be delivered once or repeatedly in multiple sessions which may be every couple of days to as infrequently as monthly.

Where appropriate as many procedures as possible are done in one anaesthetic session, for example imaging and/or blood sampling may be performed immediately before and after a therapy. If an animal is being sedated for imaging a blood sample will be taken at the same time.

What are the expected impacts and/or adverse effects for the animals during your project?

Tumour implant: tumour growth can cause issues such as weight loss and health deterioration. In long term studies, this can result in skin ulceration, pain, and in organs such as the brain and limb

bones abnormal behaviour such as impaired balance when walking or paralysis. Daily monitoring is designed to minimise the duration of any adverse effects.

Imaging: generally imaging is based on clinical practice and adverse events are extremely rare, imaging agents are usually approved for clinical use. If more novel substances that indicate biological activity (biomarkers) are used, they will have known side-effects from studies performed under other licences.

Blood sampling: frequent sampling may result in minor bruising at the sampling site, lasting a few days.

Ultrasound guided biopsy: repeated sampling could cause bruising around the tumour, and in rare cases internal bleeding due to damaging blood vessels within the tumour.

Therapy: Physical and systemic therapies can also have side effects. Ultrasound can heat skin and cause unwanted damage and in rare circumstances pain. Radiation can damage normal tissue, with acute skin damage and chronic organ damage being most common. However, for both therapies, careful delivery makes all adverse events very rare. Systemic therapies are delivered in ways informed by clinical practice or pre-clinical trials performed under other licences, so it is very rare to encounter more than mild adverse events.

The welfare impact of any adverse events is minimised by daily monitoring of animals by trained staff using published measures of pain and ill-health. Animals are humanely killed before adverse events become severe, except animals that are not recovered from anaesthesia, therefore, most will experience moderate adverse effects, mainly due to cancer 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)?

Typically, animals become less energetic, as tumours grow, and they usually show moderate adverse events which have a relatively slow onset and progression over several weeks. Their weight is usually relatively stable even up until they are humanely killed to avoid suffering. This licence places an upper limit of moderate severity in order to allow some surgical procedures and tumour growth particularly in control animals. Tumours being the cause of the majority of adverse events. Tumour growth for frequently used cell lines is relatively predicable which assists us in minimising animal suffering. Most animals will experience moderate adverse effects, except for those in pilot studies performed without recovery from general anaesthesia (<5%), where adverse effects are avoided.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In order to develop novel cancer therapies for future clinical use, pre-clinical studies are required to best mimic the complex functioning of the human body in order to understand how it responds to both disease and treatment. For example, if a tumour promotes an immune response in the host, immune cells will be generated away from the tumour site and travel to the tumour via the blood stream. Thus, the final step of pre-clinical testing still needs to be the use of mammals, often bearing tumours similar to those naturally occurring in humans, so that the safety and effectiveness of a therapy may be demonstrated in the most biologically relevant model of human beings where the natural physiological response to both the tumour and the treatment may occur.

Which non-animal alternatives did you consider for use in this project?

We have a range of theoretical models, and perform experiments using tissue mimics/phantoms, tissue from the food chain or cells outside a living host, in particular 3D tumour spheroids (clumps of cells that are more like a tumour than a flat layer of cells grown on a plastic dish) to replace animal use. Using animals is the last stage before clinical trials and is normally preceded by the above extensive non- animal testing. We are actively engaged in looking for newer alternatives such as “organs on a chip”.
We also check for recent developments via FRAME (Fund for the Replacement of Animals in Medical Experiments) and NC3Rs newsletter subscriptions.

Why were they not suitable?

Unfortunately, no animal alternatives are currently able to mimic all the effects of the complex biological environment in mammals such as the amount and physiological regulation of the blood supply, the immune system and its response to both tumour burden and treatment, such as the repair of damaged tissue.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers are based on anticipated study designs using a combination of pilot studies and more advanced studies performed using good experimental practice, and prior experience of similar studies on our expiring licence. It is important to compare groups of animals to a baseline e.g. control animals in which no treatment is given. When combining more than one treatment, it is important to compare with the individual treatments to quantify any enhanced effect accuracy. It is also useful to know if therapies simply add together or whether one enhances another so that the combined effect is greater than the sum of the individuals. Combining this with complex statistical analysis and realistic estimates of the size of therapeutic effects enables us to estimate future animal use.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

An online facility provided by NC3R (an organisation which helps us to replace, refine and reduce our animal use), called an experimental design assistant (EDA) tool was used. It allows you to design your experiments by drawing a picture of its key aspects, providing feedback and advice on how to improve it. For example, by keeping potential variables in a study as tightly controlled as possible. It provides advice on randomising and blinding (statistically important processes for avoiding bias in experiment design and data analysis) and helps you to use your estimation of expected experimental outcomes to calculate how many animals are required in a study to avoid the result being a fluke. Thus, it allows the use of the minimum number of animals while also minimising the risk of a misleading result.

In addition, imaging follow up is a much-used technique in our studies which minimises the number of animals because each provides data at multiple times instead of just one time-point. The same is true of serial blood sampling and ultrasound guided tumour biopsies.

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, often under terminal anaesthesia, will be used where appropriate at the beginning of newly designed studies, particularly to check for unexpected adverse effects. Whenever possible medicines (etc) licenced for human use will be used at clinically recommended doses to minimise harm and the associated loss of animals from studies. Similarly, physical therapy doses will be guided, where possible, by clinical treatment

regimens that are known to be more effective and safer than other options. Staying up to date with the latest research via papers and conferences is also useful in this regard.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 the smallest model we can use with equipment which is normally designed for clinical use. Where possible we use mice, but if the target tissue is too small to allow image guidance for targeting

or if the region of therapeutic effect is likely to be much larger than it, it may be necessary to use rats. Hence why we predict the use of significantly more mice than rats.

There are many cancer types that can be grown in rodents (particularly mice) and having the physiological response of the whole body to the tumour and the treatments is important for developing clinical treatments. Where possible we modify equipment in preference to using larger species. We minimise the welfare costs to the animals in the following ways:

We predict and manage adverse effects based on the experience and knowledge within our team, and our colleagues, and the published literature. Studies are ended based on the risk of animals suffering e.g. there are size limits on tumours and limits to the severity and duration of any adverse effects.

Animals are provided with an appropriate environment and stimulation including nesting material and cage furniture such as tunnels and chew sticks.
Where appropriate pilot studies are used to establish the design of subsequent experiments.

Anaesthesia/sedation and analgesia (pain relief) are used for steps which involve surgery or the potential for animals to experience pain.

Imaging follow up is a core technique in our studies which minimises the number of animals because each provides data at multiple times instead of just one time-point. The same is true of serial blood sampling and ultrasound guided tumour biopsies.

Why can’t you use animals that are less sentient?

The development of treatments for human cancer based on ultrasound requires pre-clinical testing of efficacy and safety in mammals prior to clinical trials. Where possible procedures are performed under terminal anaesthesia.

In early phase studies such as pilot studies, the bodies of animals killed at the end of other studies or meat from the human food chain will be used before progressing to studies under terminal anaesthesia. Only once these have been successful will longer term survival studies be undertaken, using mice in preference to rats.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be monitored daily, and more frequently following potentially painful procedures such as surgery. Animal technicians and scientists are trained to recognise signs and symptoms and use standardised methods of assessing their severity. Animals are humanely killed before adverse effects exceed the licence limits.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We use the NC3R Experimental Design Assistance (EDA) tool and the ARRIVE guidelines are embedded in our internal study plans. Both of these provide a framework for effective scientific communication of results.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We continually review experimental options and seek advice from our animal care staff, Named Animal Care & Welfare Officer and supporting Veterinarian as well as staying up to date with recommendations from NC3R and FRAME circulated by our Biological Services Unit, and newsletters e-mailed by those organisations.

45. The impact of inflammation on the propagation and resolution of deep vein thrombosis

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

Deep vein thrombosis, Inflammation, Thrombus resolution, Mast cells, Inflammasomes

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims at establishing inflammatory mechanisms that regulate growth of blood clot in veins, and involvement of these mechanisms in further dissolution of the clot.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Deep vein thrombosis (DVT) is a severe life-threatening disease that strikes over 60,000 people in the UK every year despite all prophylactic measures. The essence of the disease is formation of a blood clot in veins (i.e., blood vessels bringing the blood back to the heart). This clot causes pain and difficulty to walk, and can get dislodged and travel to the lungs, where it occludes one of the lung vessels leading to hardship of breathing and,

potentially, death. DVT is a third most dangerous cardiovascular disease with only myocardial infarction and stroke having higher mortality rate.
Remarkably, the number of people dying from DVT exceeds that from HIV, breast cancer and traffic accidents, combined.

Those at risk for DVT are adult individuals, people with certain types of cancer or after major surgery, or with severe systemic infection, or deprived of the ability to actively move due to any other reason. Recent studies have discovered that inflammatory factors are tightly involved in the regulation of various aspects and stages of DVT development. Moreover, in many cases, the clot cannot be surgically removed and has to be at least partially resolved by natural resources of the organism, a big part of which belongs to inflammatory mechanisms, which remain incompletely understood.

Importantly, virtually all drugs currently used to prevent or treat DVT in clinical practice inevitably cause bleeding complications in a proportion of patients, which makes identification of new and safer therapeutic approaches that would either reduce clot formation and growth or stimulate its resolution and revascularization (formation of new blood vessel inside the clot thereby restoring normal blood flow) a very important and timely research task. This will pave the way to preventing DVT in people at risk, saving lives of DVT patients, improve their quality of life and decrease heavy economic burden on health systems worldwide.

What outputs do you think you will see at the end of this project?

This study will generate important information about the impact of inflammatory mechanisms into DVT development and resolution. This information will expand the existing knowledge on the role inflammation plays in blood clotting and identify new fundamental opportunities to developing novel therapeutic approaches that would allow us to fight DVT causing little to no dangerous bleeding complications.

Who or what will benefit from these outputs, and how?

1. The immediate short-term beneficiaries of this study are colleagues and collaborators specializing in this research topic. Fundamental information originating from this project will expand our knowledge in the field and stimulate further research in this direction; this is an immediate benefit that will become evident already during the course of project implementation and PPL duration.

The following beneficiaries will likely benefit from the results of this project at the timescale beyond the lifetime of this PPL:

2. Medium-term beneficiaries:

Research community around the world, dealing with studies in the field of thrombosis. This will be based on publication of research papers and presentation of the results at scientific meetings and conferences.

3. Long-term beneficiaries:

In the longer perspective, results obtained in this project will be beneficial to both sides involved in the clinical practice, namely, physicians and patients. Identification of novel

mechanisms controlling inflammation leading to and accompanying DVT will be established, which will constitute the basis for their targeting therapeutically thus rendering anti-thrombotic therapy more safe, flexible and personalized.

How will you look to maximise the outputs of this work?

The results of the project will be published in professional scientific journals and shared with the general public through mass media. The data will be presented at both national and international scientific conferences. I will collaborate with world leading experts in the thrombosis field to ensure highest quality of the work and reliability of the obtained results. I will stay in constant contact with physicians dealing with individuals at risk for DVT and DVT patients to make sure that the research is being performed in accordance with actual clinical needs. Importantly, every effort shall be made to publish even negative data, which is very important as it saves time, efforts, resources and animals that would otherwise have been wasted in an effort to perform the same experiments.

Species and numbers of animals expected to be used

- Mice: 6200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Although any animal model has its disadvantages and none of the models ideally reproduces all aspects of human disease, we have chosen the model of flow restriction in a large vein because it is well tolerated by the animals and allows for obtaining reliable and reproducible data. Besides, we have chosen this experimental approach because: 1) over 90% of the existing preclinical data on mechanisms of DVT has been generated on mice and therefore the use of the same species is necessary to optimally plan experiments basing on existing information, and also to have the opportunity to appropriately incorporate anticipated results into current state of knowledge on the topic;
2) mice are the least sentient species that display suitably human-like anatomy of the entire cardiovascular system and veins in particular; 3) mechanisms of DVT established using mouse models are applicable to the human disease, which renders the study clinically relevant; 4) the mouse DVT model that we plan to use is very well-established and thoroughly explored so that the number of required animal as well as individual animal suffering can be minimized; 5) mice are the only species for which a wide range of genetically altered strains is already available, which minimizes the required breeding and makes mice an optimal model to study roles of single molecules in DVT.

We have chosen adult mice due to the following reasons: 1) the structure of the vessel wall in large veins in mice is similar to that of human veins and the mechanisms underlying clot formation are known to be reasonably comparable leading to the applicability of drawn conclusions to the real human disease; 2) the mouse cardiovascular system continues growing and developing after birth together with the entire organism leading to relative stabilization at the mature state after about 2-3 month, which underlies the choice of the life stage; 3) DVT develops in adult people and not in embryos or newborns, which

explains the necessity to use adult animals in order to better mimic the cohort of people mostly susceptible to DVT.

Typically, what will be done to an animal used in your project?

As mentioned above, DVT in humans frequently develops as a result of prolonged immobility, which leads to reduced blood flow speed in the veins. In our model, we will recapitulate this major factor driving the disease. DVT will be induced in some of the animals by the following procedure.

Anesthetized mice will undergo incision into the abdomen, the intestines will be moved to one side, and then the main artery (aorta) will be carefully separated from the main vein (inferior vena cava, IVC) to allow partial or full ligation of the IVC. The side and back branches of the IVC will be either closed or left open. This will be followed by repair of the abdominal wall. Then, mice will be allowed to wake up and maintained for up to 21 days in order to assess different stages of clot development.

Either prior to, or following the ligation surgery, mice may be administered different drugs or chemicals affecting inflammatory processes implicated in either DVT development or resolution, or cells participating in the inflammatory response to explore their role in the DVT process. Some mice will be administered components of bacterial wall, for example, lipopolysaccharide, to explore the mechanisms of thrombus propagation and resolution in the wake of systemic inflammation induced by bacterial infection. In some mice, DVT will be assessed by Doppler ultrasound. In some mice, blood samples may be taken in the course of experiment. The health conditions and well-being of mice will be closely monitored and in case of any unexpected deterioration in their status the animal will immediately be humanely killed. Analgesia will be used to treat any pain associated with the surgery itself. The number of animals used in experiments will be limited to minimal possible based on a special statistical test called "power calculation".

What are the expected impacts and/or adverse effects for the animals during your project?

Mice usually tolerate this surgical procedure well and quickly recover after anaesthesia. Nevertheless, the animals may lose up to about 10-15% of their body weight during the first several days post- surgery. There is a tendency for the animals to regain the weight after around a week. Mice will be provided with analgesia as required.

Other, although rare but possible adverse effects include bleeding during surgery, and/or impaired gait, reduced activity, starey coat, and/or hunched posture during the first hours post-surgery. Additionally, if oxygen levels drop too low during surgery then an involuntary reflex could be triggered, causing the animal to take deep rapid breaths. In this situation, the anaesthetic will be adjusted to improve oxygen level.

Very rarely, breakdown of the sutures or infection at the site of surgery could occur.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

80% mild

20% moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Formation of blood clot is a very complicated process affected by multiple variables, such as blood clotting system, inhibitors of different stages of the clotting cascade, fibrinolysis (destruction of the clot, a natural process counteracting clot development), factors synthesized and released by the vessel wall, components of the immune system and the inflammation machinery, and hemodynamics or blood flow geometry, for example, such factors as blood viscosity, shear stress. Multiple cell types contribute to the fragile balance of forces supporting and antagonizing thrombosis, including the variety of blood cells, endothelium, mast cells, smooth muscle cells, fibroblasts etc. It is impossible to take into consideration or predict the involvement and the relative impact of all uncountable less prominent factors that, alone or in combination, might influence thrombosis. Adequate modeling of a process of such degree of complexity in a test-tube or a flow chamber is impossible at the current stage of scientific knowledge and the modern level of technical and methodological development. Importantly, one of the primary goals of the project is identification of new targets that would be useful in restraining thrombosis propagation and stimulating its dissolution and revascularization, which is unachievable in an artificial in vitro system recapitulating only already established factors. Thus, it is absolutely necessary to use animals in this project to obtain reliable significant results that can be later translated into clinical practice.

Which non-animal alternatives did you consider for use in this project?

We will utilize a microfluidics flow chamber device (a "vessel-on-a-chip") that recapitulates basic features of a human vein. This device mimics blood flow regimens typical for veins, and contains endothelium, the cells covering any vessel from inside. It will be extensively used in this project to assess all aspects of DVT pathophysiology, which can be addressed without using animals. Another approach is isolating and culturing different types of cells (e.g., endothelium, mast cells, smooth muscle cells or leukocytes) in vitro in order to evaluate separate well-specified aspects of their functions related to DVT. In particular, we will test the effects of different factors on the ability of endothelial cells to proliferate, migrate, and form vessel-like structures to screen potential usefulness of a range of these factors in clot revascularization (growth of new blood vessels inside the thrombus. In collaboration with colleagues specializing in engineering, we will use in silico modeling of different patterns of thrombus growth and dissolution. Finally, we will utilize analysis of human material obtained from DVT patients (thrombi and blood plasma) to test certain markers and clot components without involving animals.

Why were they not suitable?

The aforementioned methods allow for studying separate aspects (e.g., single specified process, interaction between two molecules, alleged role of a separate cell type etc.) of clot development, but do not recapitulate the entire process with all it complexity. Due to this, even the term "thrombosis" is not applicable for in vitro studies thus emphasizing that such studies focus on isolated key points rather than on the process of thrombosis as a whole. However, all the listed in vitro or in silico approaches will be our first choice in all cases, in which value and reliability of results obtained using them is expected to be comparable with those anticipated using mice.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The total number of animals required to reach the goals of the current project has been evaluated based upon: 1) published data on the DVT-related research; 2) personal experience as well as data

generated in-house; 3) personal communications with research teams worldwide conducting research in the field of DVT initiation, propagation and resolution, and 4) Experimental Design Assistant (EDA), a free software developed by the NC3R.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will implement the following measures to ensure that minimal possible numbers of animals are involved. First of all, wherever possible, experiments will be performed in a blind fashion. This means that the person performing the experiment will not be aware of the administered treatment or mice genetic background. Where possible, appropriate randomization of animals in experimental groups will be used. This is a well-known method employed to obtain more objective and reliable results and thereby decrease the number of required animals. Every effort will be made to use material obtained from the same animal to address different scientific questions simultaneously, for example, tissues obtained from the same mouse will be used to measure different biological parameters thus limiting the necessity to perform additional experiments and use more animals. For this purpose, when possible, various tissues will be collected from each animal at the end of experiments; also, these tissues will be processed and stored in different conditions (e.g., as both frozen and paraffin-embedded blocks, blood drawn in different stabilizers etc.) that will allow for the use of the same material for as many scientific purposes as possible.

When possible, animals will be used as their own control. For example, when possible, clot formation in the same mouse will be assessed by Doppler ultrasound, which will allow us

to avoid using separate animal groups for each time point. For similar reasons, blood samples may be taken during the course of experiment in accordance with the existing guidelines. This approach will not only allow avoiding the use of separate experimental (and, consequently, control) groups of animals, which is already a significant reduction, but also reduces numbers of mice in each group because "paired" statistical tests used to compare results obtained from the same animal, require substantially less animals to allow for correct statistical comparison.

Where possible, a statistical method designated as "Resource Equation" will be employed to design the experiment. This method allows to limit the number of required animals to minimal possible.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

One of the most important measures allowing to reduce the number of animals is implementation of as similar as possible (ideally identical) treatment of each animal prior and during the course of experiment. All mice will be maintained in the same conditions prior to experiments, and undergo surgery according to standardized protocols (when possible, performed by the same surgeon) as well as post-operative treatment to minimize effects of environmental factors and thus improve reproducibility of the results. Mice in both control and experimental groups will be comparable in terms of age and weight to minimize inter-individual variations. For the same purpose, littermates will be used where possible. Timely planning of all experiments will be employed to ensure optimal breeding strategies are implemented to limit generation of animals to the required minimum and thereby minimize wastage. When possible, appropriate alternative in vitro and in silico approaches will be utilized to evaluate the effects of certain conditions and processes (e.g., functional status of the endothelium, degranulation of mast cells etc.) to avoid the use of animals. These alternatives (such as use of the ”vessel-on-a-chip” technology) will allow to either completely replace animals to answer the required scientific questions by an in vitro method, or the number of required animals will be substantially reduced and limited to checking and verification of selected results of in vitro screening. All the results will be constantly monitored for any inaccuracies and appropriate corrections will be introduced to the experimental design by using appropriate statistical methods for data analysis.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have chosen to use flow restriction (either complete or partial) in the large vein called inferior vena cava as a model of DVT. There are several other models, but we have decided not to use them at the moment because they provide less relevant scientific

information while being equally or less refined as compared with our model. In particular, the following alternatives exist:

- Induction of thrombosis in the inferior vena cava by chemical or mechanical denuding the endothelium. In particular, thrombosis can be induced by application of ferric chloride or mechanical pressure by a surgical clamp. In both cases, virtually the same surgery is required but endothelial denudation is not observed in human DVT and therefore the translational value of results obtained in these models is questionable and they are used in only a small part of research in the DVT field;

- Induction of thrombosis by application of electric current. Again, this model requires the same surgery, then a needle is introduced into the inferior vena cava and electric impulse is applied. This induces rapid thrombus formation, however its structure as well as other scientific parameters (damage of the vessel as a driving force of thrombosis, necessity to make a hole in the vessel that may lead to uncontrollable bleeding, instability of the resulting thrombus, which may lead to its embolization and sudden deterioration of the animal welfare) make scientific value of this model quite low and it is used by only 1-2 labs in the world;

- Induction of thrombosis by administration of a special substance called siRNA that inhibits mechanisms counteracting blood clotting. This model induces development of thrombi of unpredictable localization and at an unpredictable time point, and therefore requires very large numbers of animals to obtain more or less standardized results. Moreover, the model can manifest by severe clinical signs, such as eye injury, seizures or sudden death of the animal (if the clot is formed in the brain), which makes it the least refined one.

It should be noted that complete and partial occlusion of the IVC are two independent, although technically similar models. Thrombosis in these approaches is induced by different mechanisms (predominantly activation of tissue factor-dependent blood coagulation in complete stasis and local inflammation-like process in partial stenosis) which allows for generation of complementary results and gathering full information about the role of the investigated factor in modifying pathophysiological routes implicated in DVT development.

Why can’t you use animals that are less sentient?

Mice are used in the vast majority of pre-clinical/translational DVT research worldwide because: 1) the structure of large veins is very similar to human vessels 2) mechanisms of thrombus formation are also similar to humans, for example, thrombus structure, time kinetics, dependence of the same factors (e.g., platelet or neutrophils) 3) drugs used in clinical practice, for example, heparin, prevent DVT in mice as well. Other species used in biological research either have absolutely different vessel structure and blood composition and are virtually impossible to adequately model venous thrombosis at all (e.g., zebra fish), or are higher species than mice (for example, rats, guinea pigs, rabbits, dogs etc.). The same logic can be expanded to less mature life stages because embryos and very young mice have only developing venous system from both anatomical and functional points of view, and cannot therefore be used for modeling DVT. The time required for DVT development and, even more so, resolution, is days to weeks, which makes the use of terminal anesthesia impossible as the animal would not endure such a long time of anesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice will be handled using refined handling techniques (https://www.nc3rs.org.uk/3rs-resources/mouse-handling), and welfare score sheets will be used routinely to monitor animals for potential adverse effects. The health condition of the animals will be monitored particularly closely immediately post-surgery to ensure animals are not suffering unexpected adverse effects. Additional supportive measures will also be in place, based upon in-house experience. This includes keeping the animals warm during and after surgery, and provision of a subcutaneous injection of saline to restore liquid balance, both of which have been shown to help animals to recover after a surgery better.
Animals may also receive water-containing gels and food on the cage floor to help aid recovery. Analgesia will also be provided to help alleviate pain associated with surgery.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Maximum frequency and volume of blood withdrawal will be in accordance with LASA guidelines. Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines will be implemented to ensure appropriate planning of the project, choice of appropriate experimental design and statistical approaches, and to decrease experimental bias.
Results of this project will be published in high impact peer-reviewed scientific journals conforming with the ARRIVE2.0 guidelines provided by NC3Rs (https://www.nc3rs.org.uk/sites/default/files/2022-01/The%20IMPROVE%20Guidelines%20%28poster%29.pdf).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will regularly and thoroughly review the NC3R web site https://www.nc3rs.org.uk/, and implement any new requirements or recommendations that would appear during the course of this project. I will make sure that my research team is well aware of the news in 3Rs, which will be discussed at our lab meetings at least once a month. I will work in tight connection with our animal facilities to exchange any new information and implement new approaches to refinement. I will also monitor professional literature in the field to learn potential new statistical strategies to reduce the number of mice required for every experiment.

46. Investigating Novel Immune and Biological 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

Key words

Cancer, Oncolytic Viruses, Immunotherapy, Radiotherapy, Chemotherapy

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 primary aim of this project is to improve direct cancer-killing by using viruses as a type of immunotherapy that boosts the immune system for the treatment of melanoma and other cancers (e.g. lung, head and neck, breast, liver, brain, ovarian, pancreatic cancer) by:

- enhancing the delivery of oncolytic viruses (viruses that have been engineered to only infect and replicate in cancers) to tumours via the bloodstream, by improving our understanding of the interactions between the virus and the antibodies produced by the body as a natural defence against it.

- improving immunotherapy (therapy that harnesses/boost the body's own immune system), and our understanding of how it works, by testing combinations of oncolytic viruses (OV), immune checkpoint inhibitors (a class of therapy that awakens the body's T-cells to recognising cancers) and clinical standard of care agents (e.g. radiotherapy, chemotherapy and small molecule drugs).

The secondary aim of this work is to develop and validate a novel in vivo tumour oxygenation monitoring probe (measuring the level of oxygen present in the tumour) using diffuse reflectance spectroscopy (an instrument that measures the light reflected from the tumour), which may be used to monitor how well the therapies are working (radiotherapy in the first instance, but could also include chemotherapy, and oncolytic virus) in 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?

Our aim is to investigate, understand and improve immunovirotherapy (immunotherapy using viruses) using clinically relevant animal tumour models (that is similar to the respective human cancers) and treatment strategies, by testing combination therapies and investigating how they change the immune system to recognise the tumour cells and treating the disease. This new understanding of how immunovirotherapy and other treatment combinations work, will not only guide the next stages of clinical trial development, but will also provide further insights on how these viruses actually interact with the cancer and the immune system to combat the disease.

Hypoxia (the deprivation of oxygen supply) in tumours have been shown to enhance tumour spread and metastasis. Our aim is to validate a novel device designed for measuring tumour oxygen levels in mice. This will be used to monitor how well the therapies (including radiation, immunotherapy and chemotherapy) are working in the tumours and may lead to the development of a clinical device for monitoring tumour response and prognosis.

What outputs do you think you will see at the end of this project?

Improving virus delivery to the cancer: Currently, oncolytic viruses (OVs) are being delivered to the tumours by direct injection into the tumour itself. As this limits the number of patients that can be treated in this way, the ideal delivery system to broaden the number of patients that can be treated with OVs is by injection into the bloodstream. This may be hampered by the presence of virus neutralizing antibodies (antibodies made by the animal against the virus due to previous infection), so the virus is being inactivated in the bloodstream before it can reach the tumour to take its desired effect. The project will explore novel ways of protecting the virus from antibody neutralization (e.g. by coating virus with nanoparticles) and thus ultimately enhancing OV delivery to the tumour in cancer patients.

Combination therapy: To maximise the benefit of OV and other immunotherapies for patients, we need to optimise combination strategies incorporating other standard of care treatments and interventions, including immune checkpoint inhibitors, radiotherapy, chemotherapy, small molecule drugs. At the moment, many immunotherapies are progressing rapidly in the clinic without proper rational, scientific choice of the best combinations, nor undergone pre-clinical studies to gain mechanistic understanding that justifies their use. This project will use preclinical mouse models of advanced cancer to test strategies which maximise the immune response generated against the tumour following such combination, which ultimately harnesses the immune system against the

cancer. This data will then be used to develop and inform novel treatment approaches for patients with cancer. This will be achieved by publishing our data in peer-reviewed journals, collaborations with scientists and clinicians nationally and internationally, and development of clinical trials led by myself in my clinical role, together with clinical colleagues at the hospital. We have succeeded in this clinical translation previously, with our murine work supporting a translational trial, which in turn led to an ongoing study adding an OV to adjuvant chemoradiation following surgery for high grade glioma.

Monitoring hypoxia: This work will enable the validation of diffuse reflectance spectroscopy (special instrument that measures light reflected from the tumour) as a tool for measuring tumour hypoxia (insufficient levels of oxygen), and whether this measurement of tumour hypoxia can predict the therapeutic response to radiotherapy. This will, in turn, enable the onward development of a clinical device for monitoring tumour response and prognosis.

Who or what will benefit from these outputs, and how?

Within the timeframe of the new licence (5 years), the outputs of the work will benefit other scientists working in this research field. The advances in our knowledge will then be used to develop and inform novel treatment approaches for patients with cancer as well as contribute towards the development of a clinical device for monitoring tumour response and prognosis. In particular, this project will help us scientists understand better the complex interplay between tumour cells and the different immune cells and how to exploit this interaction therapeutically.

How will you look to maximise the outputs of this work?

Findings will be made available to other scientists and clinicians through peer-reviewed publications, presentations at national and international conferences, at meetings with established and potential collaborators, and via our website and social media outputs.

Species and numbers of animals expected to be used

- Mice: 23900

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We have chosen the least sentient animal species (mice) possible for all studies. For these type of cancer therapy experiments we need to use mammals in order to replicate tumour growth and immune system activation in humans. In the majority of experiments we will use adult mice. However in some cases there we will use juvenile mice for breast cancer studies to better reflect early onset of cancer in younger patients.

Typically, what will be done to an animal used in your project?

Most animals will receive an injection to grow a tumour. The majority of mice will receive treatment (or control) for cancer, this can include treatment through food or drink or through various types of injections for example into the blood stream or directly into the tumour. Some animals will undergo multiple treatments on the same day but care will be taken to design the experiments to use the minimum number of injections possible per day without compromising the results of the study. Some animals will undergo radiation treatments. Some animals will undergo surgery but anaesthesia and pre- and post-operative analgesia (pain relief) will be used to minimise suffering and distress. Some mice will be imaged to investigate cancer spread. All of the procedures are characterised as moderate by the Home Office. A normal experiment lasts 4-5 weeks, with treatment ongoing for approximately 2 weeks time. All experimental mice will be monitored daily. At the end of an experiment, animals will be humanely killed and samples, such as tumour, lung, spleen and blood, will be taken for processing and detailed analysis to help improve future treatments for cancer patients.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of the mice will develop tumours and will exhibit clinical signs of cancer similar to those seen in humans such as weight loss, lethargy and pain. As we are testing a variety of experimental anti-cancer agents as well as standard-of-care agents, we need the mice to have established tumours and will therefore inevitably show some or all of these clinical signs. Mice will receive treatments similar to cancer patients through oral dosing or injections. Injections might cause pain but that is not expected to be more than mild and not sustained discomfort. Different types of cancer treatment can result in weight loss. Some animals will undergo radiation treatments which might result in skin inflammation. During surgical procedures aseptic techniques will be used to avoid and minimise the likelihood of wound infection, general anaesthesia coupled with peri- and post-operative analgesia will be administered to limit the transient pain/discomfort from surgical procedures. At all times humane endpoints will be established to ensure that the mice do not suffer any more than is absolutely necessary.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The animal models will mostly mild or subthreshold in severity (98%) and will include juveniles to adults. The key characteristic of mild procedures is that any pain or suffering experienced by an animal is, at worst, only slight or transitory and minor so that the animal returns to its normal state within a short period of time.

90% of the adult animals will develop tumours in target tissues/organs and will be classified as experiencing moderate severity due to tumour formation.

All the animals involved in tumour studies will be moderate in severity (>98%). All of the the procedures are characterised as moderate by the Home Office.

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?

Extensive studies are first carried out in vitro to mimic as many as possible of the cellular processes and interactions as well as responses to therapies that we study. However, a large number of questions can only be addressed using animal models, as the immune systems in mice and humans are too complex to be recapitulated in *in vitro* (outside of the animal) experiments.

Hypoxia (insufficient levels of oxygen) in the tumour have been shown to drive tumour progression and spread. The consequences of hypoxia have been well studied in cancer cells in the *in vitro* setting.

However, to validate the Diffuse Reflectance Spectroscopy Probe (an instrument that measures the light reflected from the tumour) use as a sensing tool, it is essential to re-create the hypoxic tumour microenvironment (the ecosystem that surrounds the tumour inside the body)– particularly in relation to hypoxia and radiotherapy treatment. As such, use of animal tumours is unavoidable for the purpose of this investigation.

Which non-animal alternatives did you consider for use in this project?

Whenever possible we will use *in vitro* models (cancer cells grown the in the lab) that are both 2D (grown as a flat layer) and 3D, as replacements for animals wherever possible. In addition, our laboratory makes extensive use of *in silico* models (using computer modelling and simulation from human cancer databases to understand the biology and behaviour of the cancer) as well as *ex vivo* techniques (experiments performed in the lab from animal tissues). Furthermore, we use human patient samples in *in vitro* immune assays (using donor blood and isolating the immune cells to study their interactions with cancer cells) to both substitute and compliment our *in vivo* work in an effort to establish *in vivo* replacements as often as possible.

Why were they not suitable?

In vitro systems are not able to recapitulate all of the effects of an intact immune system. Therefore, it is unavoidable that some experiments will need to be performed in tumour-bearing animals.

The absence of a functional immune system which will mimic tumour formation, progression and response to therapeutic intervention as observed in humans, makes the other systems unsuitable for these studies. Since our research focus is on how tumour cells communicate with immune cells and how they are able to trigger an immune response upon therapies, the use of animals is well-suited to answer these questions and help design effective therapeutic options for cancer treatments. This complex interaction

between the immune system, the tumour cells and the different therapies we are combining, cannot be studied using human patient 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?

The numbers of mice have been estimated based on our experience from a previous project licence for the same protocols combined with the use of statistical tools to calculate optimal and minimum number of animals for each experiment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Pilot studies will be carried out to test any new tumour models or toxicity testing if these methods have not been established in the research group. Experimental Design Assistant (EDA) from NC3Rs is a very useful tool to design experimental studies. It utilises a step-wise approach in the experimental planning phase and provides feedback when needed to incorporate the correct animal numbers to generate statistically useful data, which further reduces the number of animals employed in each study.

Literature reviews will help evaluate if similar experiments have already been done by other research groups to avoid duplication of data and thereby avoid unnecessary usage of animals for repetitive studies.

In addition, we will ensure excellence in the conduct of animal procedures by adherence to standard protocols and good training of investigators to reduce the need for unnecessary repetition of studies. We have optimised group sizes to ensure that study objectives are met, without including unnecessarily large numbers of animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We are constantly trying to refine our work and minimise use and number of animals. We ensure that we are using optimum group sizes and hence, minimum number of mice. We take care to have refined techniques to reduce number of mice and experimental variability thus reducing the need for repeat experiments. We also ensure that each experiment is appropriately and maximally analysed to obtain maximum amount of information. In addition, we plan and coordinate experiments to harvest and collect as many organs for analysis as possible, including lymph nodes, spleens and blood in addition to the primary tumours, to fully analyse the immune and tumour cell responses from each experiment.

Furthermore, we use non-invasive imaging (where appropriate) to monitor tumour growth and spread. This may minimise the cohort size because without imaging, larger cohorts of

animals may be needed to be culled at different timepoints of the experiment to determine tumour growth and metastasis.

Additionally using tumour biopsy enables us to sample the same tumour pre- post and during treatment. This reduces the need to setup large cohorts of animals to be sacrificed at different time points during treatment regimes.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used in this project. Mice are the lowest species that are appropriate for drug development studies and for studies heavily based on the response and action of the immune system. Our studies involve analyses of complex interactions between immune cells, cancer cells, and other supporting cells around and within the tumour (tumour microenvironment). We use subcutaneous injections of tumour cells as well as injections into the orthotopic sites (organs or tissues where the tumour cell were originally isolated from such as skin, lung, pancreas and liver) and relevant metastatic sites (such as brain and liver) to study the complex interactions between tumour cells and immune cells. For the injection of tumour cells into the liver and pancreas, this may be performed using ultrasound guidance as this would have the benefit of avoiding abdominal surgery and associated complications. There is also a much reduced risk of wound infection and no additional adverse effect with ultrasound-guided injections. We almost exclusively use immunocompetent mouse strains (having a fully functional immune system) instead of genetically altered immunodeficient strains (having defects in one or more immune components in the immune system).

Why can’t you use animals that are less sentient?

Mice are the lowest species that are appropriate for studying tumour growth and responses to different therapies and is heavily based on the response and action of the immune system. Our studies involve analyses of complex interactions between immune cells, cancer cells and other cells around and within the tumour.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The use of the proposed cancer models are designed to provide maximum information from the minimum number of animals compatible with statistical requirements. This will be achieved by (i) using well established cancer model systems with predictable growth, (ii) obtaining multiple measurements of tumour development, (iii) analysing a number of parameters from individual experiments.

We make every effort to ensure the optimal welfare of the mice by (a) only having well trained staff undertake the studies, (b) using non-invasive methods such as ultrasound, bioluminescence imaging to monitor tumour growth (the cancer cells have been altered to emit light in the presence of an injected agent (luciferin) as they grow bigger which can the be detected and correlated to determine the tumour size) and response to therapy, (c) using statistical power analysis to ensure that optimal number of mice in each experiment is used, and (d) taking care to ensure that each experiment is analysed in depth (often by multiple researchers working on different parts of the project) and that the maximum amount of information is gathered.

Animals will be constantly monitored post procedures and therapeutic interventions will be administered with the minimal number of injections needed for an effective treatment option.

The use of genetically altered animal models which restrict expression of genes to a particular cell type, may reduce the adverse effects compared to genetically altered animal models where the expression of genes is restricted in the whole animal.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow NC3Rs resources for practical guidelines and ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines to report our animal research to ensure that good detail is reported.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We maintain the highest levels of care and welfare. All our activities are covered by standard operating procedures. Our BSU produces quarterly newsletter that will keep us informed of any new information relevant to animal research, including 3Rs. Our laboratory manager attends our establishment’s BSU user meetings as a representative of our team. The minutes of BSU user meetings which includes minutes from the Named Persons meetings and Technician Discussion Forums is shared with the team. Every team member also receives NC3Rs newsletter and publications which will inform them of any new information. Team members may attend national or international conferences to stay informed of advances in the field. The principles of 3Rs is implemented in our animal work.

47. Mechanisms underlying skeletal muscle maintenance

Project duration

4 years 2 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

Skeletal muscle, Muscle disease, Genetic condition, 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?

This research aims at understanding the mechanisms that regulate the maintenance of skeletal muscles by focusing on the role of proteins associated with inherited muscle disease in humans, so- called “myofibrillar myopathies”. We will use mice that carry mutations in these proteins and therefore display symptoms similar to those seen in human patients. We will test the efficacy of repurposing a common drug to treat the muscle weakness in these 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?

Understanding the factors and mechanisms that contribute to muscle maintenance and adaptation is essential to design treatments that will help prevent muscle loss because of injury, disease, environmental factors or old age. Previous research has exposed two new genetic mutations as a cause of muscle disease and new opportunities for intervention. We will focus on characterisation of the mechanisms leading to disease and the application of a pharmacological treatment as an intervention. If effective, this treatment will have the potential to alleviate symptoms caused not just by these mutations, but also other genetic conditions with a similar diagnosis.

What outputs do you think you will see at the end of this project?

A better understanding of the function of proteins that are essential to maintain muscle structure and function.

A detailed characterization of the disease that develops in a mouse model of human inherited muscle disease.

An in-depth assessment of the effectiveness of a pharmacological treatment to alleviate symptoms in a mouse model of human inherited muscle disease.

Who or what will benefit from these outputs, and how?

The outputs from this research will be a particular interest to:

Patients who suffer from these rare diseases, their families and the clinicians that attempt to manage these incurable disorders. Since we are repurposing a well-known anti-diabetic medicine, the results from our proof-of-concept study could be translated much quicker because there will be no need to test the safety of this compound in clinical trials.

The scientific community focused on neuromuscular research. In the short term, we will learn how the muscle becomes smaller and weaker when certain key proteins are lost due to genetic mutations in mice. In the process, we will describe the mechanism contributing to the muscle disease, that is, what pathways have been disrupted because of mutations in those proteins. Since these proteins are also found to be mutated in human patients, the information gathered from characterisations in mice will inform the human condition.

The results from the drug treatment will be completed and reported, and we anticipate the conclusions will be of particular interest to biotechnology and pharmaceutical companies interested in developing drugs for rare muscle disorders.

How will you look to maximise the outputs of this work?

We will disseminate our results at scientific meetings that focus on muscle disease and in publications in Open Access journals. We have long-standing collaborations with clinicians working on neuromuscular diseases in research institutes in the UK and abroad. Under our previous licence, these collaborations have produced high impact publications on the

diagnosis of a rare muscle disease. We use the ARRIVE guidelines to optimise the reporting of information.

Species and numbers of animals expected to be used

- Mice: 250 mice

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse has been shown to be an excellent model at replicating the symptoms observed in human muscle disease. In the case of mutations in the proteins of interest in this project, adult mice best represent the condition because symptoms arise from the use of muscle in adulthood and not from a young age.

Typically, what will be done to an animal used in your project?

Some animals will be used for tissue collection for histological characterisations.
Some animals will be challenged by well-established behavioural tests to measure whole muscle function. These tests are non-invasive tests such as grip strength. A grip strength test typically takes less than 15 seconds. Three repeats will be done.

Some animals will be exposed to a procedure involving a muscle in the hind limb to test the expression of genes of interest to help us understand their function in adult skeletal muscle. Each animal will receive a maximum of two injections, one per leg.

What are the expected impacts and/or adverse effects for the animals during your project?

The two neuromuscular mouse lines used in this licence can develop symptoms like a mild human muscle disease. These mice do not develop severe symptoms at any age and there are no overt signs of pain or distress, based on non-invasive examination using the Mouse Grimace Scale (https://www.nc3rs.org.uk/3rs-resources/grimace-scales/grimace-scale-mouse).

The first mouse line is indistinguishable from the control mice. It has a very mild muscle phenotype that affects a small group of muscles.

The second mouse line is characterised by muscles of smaller size than those of control mice. This contributes to the whole animal being smaller and lighter.
The procedure of delivering a gene of interest to mouse can cause slight temporary discomfort lasting 5 to 10 minutes. Mice recover normal mobility within that period of time and a pain killer is provided before the intervention.

Animals will receive drug treatment injections in the abdominal region daily over three weeks and afterwards they will be subjected to a simple and quick grip strength test. These

interventions are not expected to provoke any adverse health issues other than the temporal discomfort caused by a less than two-minute injection or the 5-10 minutes behavioural test.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severity levels will be mild and moderate. Approximately 50% of the mice will experience a mild level and 50% a moderate level.

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?

No other cellular system described to date can effectively model muscle disease or respond to mechanical loads with the same effects as the adult muscle in a live animal.

Which non-animal alternatives did you consider for use in this project?

Whenever possible, we use a muscle cell line that we culture in vitro. We have developed cell lines that contain the same mutations identified in human patients and we are now able to undertake characterisations in vitro in parallel to the mouse tests. For example, these cell lines are suitable for measuring rates of production and breakdown of proteins in detail. These cell lines were generated from mice, which allow us to share experimental resources such as antibodies for characterizations in vivo and in vitro.

Why were they not suitable?

Muscle growth in response to mechanical loads is not a physiological response reported in flies or worms. Most of the changes identified in adult muscles in myofibrillar myopathies cannot be replicated in cell lines. To measure the effectiveness of drug treatments, quantifications of those changes in an animal model are necessary. The three-dimensional muscle structure required to support the weight of an organism only forms in vivo, it cannot be fully replicated in a tissue culture dish.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design

studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

To obtain quantitative data we use the Experimental Design Assistant from the NC3R (https://eda.nc3rs.org.uk). This resource allows us to build an experimental design that includes calculations of the optimal number of animals required for each experiment. For qualitative data, for example when seeking information regarding the precise location of a particular protein within a tissue, generally very few mice are required that depending on the type of protein to be examined, 1 to 3 mice are sufficient.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The Experimental Design Assistant from the NC3Rs' website was used to estimate the minimum number of animals required to obtain reliable results for this project. To obtain the optimal sample size, we input information from previous investigations to perform calculations using this resource.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We reduce variability by matching sex, age and genetic background in all measurements. For histological measurements, we section the muscle at three levels and use an average from the three measurements. Variability is also reduced by consistency in the timing, dose and route of administration of the drug. Likewise, electroporation conditions (volume, concentration, muscle and current) are kept identical.

Our mouse lines have been archived as frozen sperm in mutant mouse repository centres so that we do not need to breed live mice unnecessarily.

Histology is performed at the end of the protocol, therefore the same animal will provide data from in vivo tests like the grip strength test and from histological measurements post-mortem. Tissue slides are also kept in a freezer long term in case we want to go back and examine them again later once new information on muscle disease has been identified.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 mouse mutant lines that model rare muscle disorders in humans. The mouse lines carry mutations in the same genes that cause human disease and are the best possible models we can use to characterize the disease and inform the human condition. There are no alternative animal or in vitro models to study these conditions.

To study new muscle proteins we use a methodology that requires a single muscle. This methodology has clear advantages over the classical approach of building an animal carrying a gene (a transgene) to express it in the whole individual. Expressing the gene in a single muscle focalises any potentially harmful effect in a single muscle, which has much less impact on the overall welfare of the animal.

A straightforward method to measure the muscle strength of mice will be used. The grip strength test requires a brief period of acclimatisation in the experimental room prior to the test. It involves gentle manipulation of the animal, comparable to that required for weighing a mouse using a balance.

Why can’t you use animals that are less sentient?

We must use an animal model that is similar in as many aspects as possible to the human anatomy, physiology and disease. We have created zebrafish that are mutated in the genes of interest for this project, but these zebrafish did not show muscle disease and therefore were not considered suitable models.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The delivery of genes of interest into muscles has been refined and made more efficient by selection of a readily accessible muscle, improvement of the injection technique and improving absorption by a
pre-treatment that facilitates the spread of fluids through tissues.

Administration to animals of painkillers has been implemented to prevent any discomfort. An animal welfare scoring sheet is used to assist monitoring after completion of the procedure.

A less stressful route for the repeated administration of drugs has been selected. Rather than intramuscular injections, which are likely to cause muscle inflammation and muscle damage, we will perform intraperitoneal injections in the abdominal region.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We make use of online resources at NC3Rs (https://www.nc3rs.org.uk/our-resources) and relevant methodological publications in the literature. We will be using the PREPARE guidelines (https://norecopa.no/prepare/9-test-substances-and-procedures/9a/general-principles/) to build a check list of the information that we will provide to the animal facility regarding the substances that will be administered. For reporting purposes, we follow the ARRIVE guidelines (https://arriveguidelines.org).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

During the course of previous and current licences, we have researched and kept up to date with the most refined methods via online resources from the NC3Rs (https://www.nc3rs.org.uk/our-resources), relevant methodological publications in the literature, as well as frequent discussions on best practice with other colleagues working with mice and the head of our animal facility. The routinely run meetings of facility users is also a very effective platform to disseminate new resources available.

48. Novel treatments for vascular 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

Coronary, Vascular, Disease, Stent, Graft

Animal types	Life stages
Sheep	adult, juvenile
Pigs	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To assess the effectiveness of novel interventions for the treatment of coronary-vascular disease and where appropriate, to generate the data required to obtain approval for progression into first in human clinical trials.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Coronary-vascular disease(C-VD) is the most common cause of death in the UK and the developed world. Sufferers are restricted in their ability to undertake normal physical activity and are at heightened risk of sudden death, heart attacks, strokes and limb ischemia, which in severe cases can necessitate amputation. All but the mildest cases of C-VD are treated surgically either by angioplasty (the stretching of the blood vessel to

increase it size), with or without the placement of an intravascular stent (a device that prevents the vessel shrinking back) or by coronary by-pass surgery (the replacement of the damaged vessel with a vascular graft). Whilst these procedures are highly effective at resolving the immediate impact of C-VD by restoring blood flow, over time a high proportion of treated vessels become blocked by blood clots or undergo severe narrowing or restenosis, putting the patient's health at further risk. As a result, the annual readmission rate for patients treated for C-VD is around 9% and a high proportion will require further interventive treatments. Consequently, there is an urgent need to develop improved treatments that either avoid or reduce the occurrence of late thrombosis and restenosis to improve the clinical outcomes of C-VD patients.

To address the issue of late blood clots and the narrowing of grafts and stents, scientists and clinicians are working to develop improved treatments including drugs, gene or cell based therapies, tissue engineered grafts, and stents that are either bio-compatible, drug eluting, or bio-resorbable. Before such innovative treatments can be introduced into clinical practice it is essential to demonstrate that they are both safe and effective. The purpose of the outlined work is to facilitate this evaluation process and, for promising interventions, to generate the data needed to support applications for first in human clinical trials.

What outputs do you think you will see at the end of this project?

The primary output of this project will be data on the performance, safety and efficacy of novel interventions/products for the treatment of vascular diseases (e.g. novel designs of stents, grafts & valves). The data generated will be used to support applications to translate promising interventions/products into first in human clinical trials and will also form the basis of publications in peer reviewed scientific journals.

Who or what will benefit from these outputs, and how?

The long-term beneficiaries of the work will be patients suffering from vascular disease through the introduction, into routine clinical practice, of more effective interventions developed and assessed in the outlined studies. In the short term the work will be of benefit to medical companies and scientist developing interventions/products for use in the treatment of vascular disease. In the medium term the publication of the research findings generated will benefit the career development of the scientists involved in the studies.

How will you look to maximise the outputs of this work?

The data generated will be used to support applications for first in human trials of successful interventions/ products and will published in peer reviewed scientific journals and presented at national and international medical conferences.

Species and numbers of animals expected to be used

- Sheep: 60
- Pigs: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The studies will be undertaken using juvenile pigs and adult sheep as their cardiovascular system closely resembles that of humans in both structure and size, thus enabling surgical procedures to be undertaken in a manner that closely replicates those performed on humans. In addition, well characterised models of coronary vascular disease are established in these species. Consequently, they provide models that enable the effectiveness and safety of drugs, treatments, devices and interventions, intended for used in human patients suffering from vascular disease, to be determined. The majority of studies will be undertaken in juvenile pigs however, for long term studies adult sheep will be used preferentially to avoid any complications caused by growth during the study period.

Typically, what will be done to an animal used in your project?

The animals used for these studies will be obtained from commercial suppliers at least one week in advance of surgery. All animals will be group housed throughout the study. During the first week the animals will be habituated to human contact and trained to enter the weighing crate and transport trolly in order to minimise handling stress. In most instances, the study will involve two surgical procedures conducted under general anaesthesia. During the first procedure, which is relatively minor, a balloon catheter will be used to induce a vascular lesion. The catheter will be inserted at a peripheral site and guided into position within the target vessel using non-invasive imaging. The balloon will then be inflated to stretch the vessel before removing it. Following the procedure, all animals are expected to make an uneventful recovery and to resume normal behaviour within a few hours. During the second procedure, which will take place a few weeks later, a repair will be performed upon the damaged vessel. On the day of surgery, the animal will be moved to the surgical suite in the transport trolly and will remain in the trolly during anaesthetic induction. The animal will be intubated and mechanical ventilation established before moving to the operating theatre. The animal will be mechanically ventilated and maintained at a deep plane of general anaesthesia throughout the procedure. Animals may be imaged non-invasively before, during and after the surgical procedure. Surgery will be performed by specialist cardiac surgeons supported by specialist in all relevant clinical areas including perfusionists, veterinary anaesthetists, radiographers and theatre nurses. The procedure undertaken will depend upon the nature of the intervention but could include the placement of an intravascular stent, the delivery of a drug, gene or cell based therapy or the insertion of a novel graft or patch.

Procedures to insertion a grafts or patches will be performed under bypass surgery conducted under neuromuscular blockade. Upon recovery, all animals will be given pain controlling drugs, under the direction of a specialist veterinary anaesthetist, until no signs of pain are detectable. Animals may be allowed to remain alive for up to one year during which time they will be periodically imaged under general anaesthesia. At the end of the study period the animals will be terminally anaesthetised, imaged and killed to enable tissue samples to be collected for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

All the models used in this study require the animals to undergo at least one surgical procedure under general anaesthesia. Following the procedure, all animals will be given analgesic drugs to control pain, which will be maintained until no overt signs of pain are detectable. The procedure to induce a vascular lesion is relatively minor and animals are expected to resume normal behaviour within a few hours of recovery and are not expected to develop any overt signs of suffering. The impact of the vascular repair procedure will vary according to the method of used. For repairs undertaken by the placement of a stent, a procedure involving minimal tissue damage, the animals are expected to recover uneventfully and to resume normal behaviour within a few hours. For repairs involving the surgical placement of a graft or patch, the recovery period will be more protracted but nevertheless the animals are expected to resume normal behaviour within 48 hours. Following the surgical procedure, the animals may undergo non-invasive imaging under general anaesthesia at several time point, however recovery from these is expected to be uneventful and animals are expected to resume normal behaviour within a few hours. Following vascular repair, all animals are expected to continue to grow and behave normally throughout the duration of the study.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

30% Non recovery

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?

The outlined studies are translational and aim to provide the data required to progress effective treatments and intervention for coronary vascular disease into clinical trials. It is not possible to achieve this objective without using animals as only data generated using a representative animal model will suffice to meet the criteria of the regulator responsible for authorising clinical trials.

Which non-animal alternatives did you consider for use in this project?

Non-animal models are not appropriate for these studies as only data generated using a representative animal model will suffice to meet the requirements of the regulators responsible for approving human clinical trials however, where appropriate, non-animal

studies will be used to assess the safety of the interventions/devices prior to animal testing.

Why were they not suitable?

Non-animal models are not appropriate for these studies as only data generated using a representative animal model will suffice to meet the requirements of the regulators responsible for approving clinical trials.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimate for the number of animals required is based on the throughput of work undertaken over the previous five years under my existing PPL, which covers work leading up to the current application, taking into consideration current and predicted funding. All studies will be designed with the assistance of my institute's bio-statistician, drawing on data from previous related studies to determine group size.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

My research group has considerable experience in designing studies and will work closely with my institutes bio-statistician to ensure that only the minimum number of animals are used to obtain the data required to determine the safety and efficacy of the intervention. The outlined animal models were developed by my research group and have been carefully refined to minimise the associated adverse effects whilst still yielding the required data. Data generated previously using these models will be used to calculate the group size needed for the outlined studies. All studies will use robust clinical outcome measures based on established biochemical markers or data obtained by serial non-invasive imaging.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

It is to be expected that for most studies relevant data sets will already exist to enable the experimental group size to be determined. If relevant data sets don’t exist, we will look initially to generate these using bio-banked tissue from previous studies however, if this is not possible, pilot studies will be undertaken to determine the group size needed to assess intervention for which no relevant data set exist.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The outlined studies require an established model of coronary vascular disease based in an animal with a thoracic anatomy and physical size that enables the surgical procedures, conducted routinely on humans for the treatment of coronary vascular disease, to be performed in a manner that closely replicates that used clinically. These criteria are best met using juvenile pigs and adult sheep.

Juvenile pigs will be used for the majority of studies, as they can be readily obtained at all times of the year and have been used extensively for previous translational cardiovascular research. Mature adult sheep will be used for some long-term studies, to avoid complications cause by growth, or when the anatomical arrangement or the size of the relevant blood vessels provides a more representative model.

The model will be induced by the over inflation of a balloon catheter, inserted into a peripheral vessel and guided into position using non-invasive imaging whilst the animal is maintained under general anaesthesia. Animals are expected to make an uneventful recovery from this procedure and are not expected to develop any overt signs of vascular disease.

Around a month later, the animal will undergo a procedure, performed under general anaesthesia to repair the vascular injury either using an intravascular device or using a surgically implanted vascular graft. All surgical procedures will be conducted by specialist cardiovascular surgeons working in line with NHS best clinical practice. All anaesthetic and analgesic regimes will be undertaken in line with best veterinary practice.

To assess the effectiveness of the intervention, animals will undergo non-invasive imaging at several time points during the study under general anaesthesia. At the end of the study period the animal will be humanely killed to enable tissues to be harvested for analysis.

Why can’t you use animals that are less sentient?

It is not possible to perform these studies in less sentient species as only medium sized mammals have the anatomy, physiology and size required to undertake the procedures and to provide representative models that meet the requirements of the regulators responsible for approving the translation of treatments and interventions into human clinical trials.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The models used for the outlined studies have been developed and extensively refined by my research group to produce a representative model that has minimum impact on the

wellbeing of the animals. All procedures are conducted under general anaesthesia by a highly experience team including cardiovascular surgeons, veterinary anaesthetists, perfusionists, radiographers and theatre nurses.

The animal care staff, looking after the animals, are highly experienced in post-surgical care. All animals are given post operative pain relief, under the guidance of a specialist veterinary anaesthetist, which is maintained until no overt signs of suffering are detectable.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All studies will be undertaken at GLP comparable standards. All surgical procedures will be performed to NHS best clinical standards, which either meet or exceed those recommended by LASA. All studies will be designed with reference to NC3Rs recommendations.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Staying current with developments in the field is an essential part of all research programmes, which includes 3Rs advances. My institute is strongly committed to the 3Rs and actively promotes the uptake of new initiatives. All studies are extensively discussed, including the 3Rs, during pre-study meetings, which are attended by representatives from the surgical, anaesthetic and animals care teams, and every opportunity is taken to incorporate improvements that will benefit the welfare of the animals.

49. Pharmacokinetics of Novel Developmental Drugs

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

Pharmacokinetics, Drug exposure

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 assess the blood and tissue levels over a time course of 1novel drugs following treatment administered by a suitable route.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Pharmacokinetics is term used to describe the study of the bodily absorption, distribution, chemical processing and removal (excretion) of drugs. Understanding the way in which animals process drugs is absolutely fundamental to the drug development process. A great deal of effort is made to optimise the chemical and physical characteristics of developmental drugs to ensure that there will be sufficient concentration and exposure to that drug in the tissues of interest to provide the optimal clinical benefit.

A great deal of the pharmacokinetic optimisation is performed without the need for animals. The physical and chemical properties (such as molecular size, solubility in water or fats, and electronic charge) are measured and compared to guidance that has been generated over many years (termed *in silico*). Compounds are tested for stability in a variety of challenging environments including stability in plasma and resistance to being broken down by liver enzymes. The potential for the developmental compound to be taken up through the gut following oral administration can also be assessed using 'cell culture' where layers of human gut cells are grown on a membrane and the transport of drugs across that membrane measured.

Whilst *in vitro* non-animal and computer models described above, are highly predictive and prevent the advancement of many compounds before they are tested on animals, currently there are no *in vitro* non-animal models that can fully replace animals in the assessment of pharmacokinetics due to the complexity of a complete animals with multiple interacting systems.

A pipeline of drugs in development is required in many areas of great medical need either because there are no drugs to treat the condition or the drugs that are available are sub-optimal. Examples of areas of great medical need include, drug resistant cancers, cardiac and lung disease, chronic renal disease, type 1 and type 2 diabetes and antimicrobial resistant infections.

Such novel compounds may:

Demonstrate enhanced potency or lowered toxicity and thereby improve the therapeutic outcome of patients.

Prove an invaluable option for the treatment of cancers have inherent or acquired resistance to currently available oncology agents.

Provide an invaluable therapeutic option for diseases for which there are either no, or an extremely limited number of treatments (e.g. Fragile-X or liposomal storage disease).

To provide an independent view of the efficacy of novel drugs, such that clinicians and directors of clinical trials have sufficient data in their pre-clinical submission to present to ethics and regulatory bodies and can use the drug optimally and safely.

To ensure that combined treatments are effective.

As the basis to decide whether a putative compound is effective and suitable for further testing and subsequent entry into clinical development.

Provide data to support licencing applications for regulatory agencies such as the FDA and EMA

What outputs do you think you will see at the end of this project?

The data generated will be in the form of drug levels (pharmacokinetic profiles) where the concentration of drug is measured at multiple timepoints in one or more tissues (blood, lungs, brain, liver and urine as examples). The data will allow calculation of the maximum concentration in tissues, the rate of clearance of drug and the overall exposure to the test

compound. The pharmacokinetic data will be used to confirm that there is sufficient drug at the disease site to have a clinical effect whilst ensuring the exposure is below that which would cause toxicity.

The majority of the scientific outputs of this licence will be either provided directly to the sponsor and be considered proprietary information or used to progress internal drug discovery programs. The data will be used to inform the drug development process. Data will only be placed within the public domain following agreement with the sponsor or when deemed appropriate from a commercial perspective, when the compound has been adequately characterised, and relevant patents sought and secured.

When a decision is made to place the data in the public domain, this will be achieved in the following ways: (i) publication in internationally prestigious peer-reviewed journals; (ii) presentations at national and international conferences.

Who or what will benefit from these outputs, and how?

The majority of the scientific outputs of this licence will be either provided directly to the sponsor and be considered proprietary information so in the majority of cases will not be made available more widely to the scientific community.

This work has implications for the sponsor and to inform internal discovery and development programs and is essential in the decision process of drug development. The information from this research is unlikely be directly submitted to ethics committees or to regulatory authorities (e.g. FDA, EMEA), but may also be of interest to other translational researchers within the field. These studies are not required to be run to GLP standards, as they are early in the drug discovery pathway.

Given the severity of the diseases and the importance of pharmacokinetic data, these studies will be of clear and direct benefit to the sponsor in the short term and patients both in hospitals and the community or animals in the longer term.

In the short-term, this licence will be valuable to the sponsors as it will inform decisions resulting in development being halted due to unfavourable properties, allowing resources to be more appropriately deployed, or compounds progressing to more advanced studies, ultimately leading to additional therapeutics being added to the drug development pipeline.

In the medium term, high quality compounds will enter the drug development pipeline. Wherever possible the compounds have already been de-risked as they will have suitable pharmacokinetic properties.

In the long term, this licence will lead to additional drugs being registered for the treatment of humans or animals. There is a critical need for new drugs to treat disease where current therapies are sub-optimal or where there are no therapeutic options.

The ultimate benefit will be less morbidity and lower mortality due to disease in humans and animals.

How will you look to maximise the outputs of this work?

The protocols in this application will mainly be used to provide a service to sponsors including pharmaceutical and biotech companies and academia, as such any refinements generated during the project will benefit all sponsors. In addition, any 3Rs benefits identified in the study will be widely disseminated at conferences or publications.

Data generated in the models will be used to generate Intellectual property (IP) often termed patents, which will be available to the scientific community. Following securing IP data will be published as presentations, posters or manuscripts.

Species and numbers of animals expected to be used

- Mice: 5000
- Rats: 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.

Adult mice and rats (6-12weeks old) will be used in this project. These animal species and life stage have been selected as there is a large body of scientific data available using these species.

Pharmacokinetic data generated in mice and rats has been extensively used in selection of appropriate lead candidate and compound optimisation studies.

Typically, what will be done to an animal used in your project?

In most cases this will be the first time a novel compound has been injected into an animal to measure drug levels (*in vivo* pharmacokinetics) assessed but on occasions several rounds of PK may be required.

In most cases animals will be used in these studies immediately following acclimatization. In a small number of cases where test drugs are administered by the oral route animals will have food withdrawn pre-treatment. Food withdrawal will be for up to 18h for both mice and rats.

Either mice or rats will be administered the test article at an appropriate dose via an appropriate administration route on a one or more occasions, up to three times daily. The group size and number of groups will be the minimum number considered acceptable for the purpose based on generally accepted pharmacokinetic practice. I

Blood samples will be collected by needle pricks to superficial veins (venipuncture) or temporary tail cannulation. In some cases where tissue levels are required, all sampling times will be terminal to allow organ collection, To allow easier collection of blood from surface veins animals will be placed into a warming chamber at 38C for up to 10 minutes to dilate the veins.

Typically up to 6 to 7 in-life micro-samples will be collected by venipuncture in mice or rats.

Pharmacokinetic studies can be of multiple designs but in most cases blood samples will either be collected sequentially from a vein or samples will be terminal.

In a small number of cases where it is important to collect urine and faeces samples to measure drug passed by these routes animals will be singly housed for up to 24h in metabolic cages. Rather than having the base of cage covered in bedding material metabolic cages only have a metal grid base to allow the urine and faeces to drop into collection vials below the base. Use of metabolic cages will be limited as temporary single housing and a grid flooring will cause stress to the animals.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals will suffer a small degree of stress due to restraint and administration of the treatment and collection of blood. Some additional stress will be caused in studies where withdrawal of food or being placed in metabolic cages is required.

Treatment administration and blood collection should be completed in a few seconds before animals are returned to their home cage. In the majority of cases there should be no adverse effects of the treatment administered as all will have been previously been screened for tolerability at the dose levels administered.

In a small number of cases, mild adverse effects might occur including injection site reactions, changes in respiratory rate or subdued behaviour.

In very rare cases, unpredictable severe adverse effects might occur that were not observed during tolerability assessment.

Expected severity categories and the 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 these studies more than 90% of animals should experience mild severity.

Up to 10% of animals might suffer moderate severity due to a combination of being handled on multiple occasions, food withdrawal, housing in metabolic cages and treatment plus some mild adverse effects following treatment.

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 extensive panel of non-animal alternatives such as *in vitro* models and computer modelling are used to identify and select the most suitable compounds before testing in animals.

Whilst *in vitro* non-animal and computer models are highly predictive and stop the development of many compounds before they are tested on animals, currently there are no *in vitro* non-animal models that can fully replace animals due to the complexity of complete animals with multiple interacting systems. In addition, predictions of tissue distribution and clearance generated using *in vitro* data can at times be inaccurate which could lead to sub-optimal compounds progressing to efficacy studies in disease models.

Which non-animal alternatives did you consider for use in this project?

Prior to initiating any animal experiments, all test drugs will have successfully completed a wide variety of non-animal tests and *in silico* simulations including proof that the drug can inhibit an essential process in the target clinical disease. *In vitro* and *in silico* tests can ensure that only the most potent inhibitors progress to animal testing.

Following confirmation that the disease target is inhibited the compounds would be screened *in vitro* for toxicity using appropriate cell lines and *in silico* predictions.

Multiple tests would be performed *in vitro* to confirm sufficient stability in the body, for example, stability in the presence of plasma, stability in the presence discrete liver enzymes or whole liver cells.

Additional more specific tests such as the ability to cross the gut cell wall using tissue cultured cells would be performed as appropriate dependent on the class of drug being developed and the route administered.

Why were they not suitable?

The *in vitro* and *in silico* tests are highly informative and act as an efficient gate to prevent poorly performing candidate compounds progressing. Unfortunately, whilst these *in vitro* and *in silico* assessments are effective screening tools they are not able to provide the precision necessary to identify the compounds with the most appropriate pharmacokinetics. In addition, it is very difficult to accurately predict tissue distribution and clearance other than by administering to an animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The experimental designs and analytical methods used in this project have been scrutinised by statisticians and pharmacologists to ensure good quality data will be obtained with the minimum number of animals and appropriate statistical tests are applied.

Extensive literature searches will be carried out prior to any study to ensure best study design and method are being used as well as to check the work has not already been carried out.

Where possible we will collect multiple samples from a single animal to reduce the number of animals required. In addition where feasible we will combine multiple drugs into a cassette to reduce animal usage.

The exact number of animals required will be dependent on requests from sponsors.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Multiple factors were considered during the experimental design phase including the use of microsamples to allow sequential bleeds from mouse tail veins (in parallel, the drug analysis methods will require modification to permit the use of small sample volumes whilst maintaining an appropriate limit of detection). By transitioning to microsamples the overall number of mice required for a study is reduced from 18 to 6 mice.

Where feasible blood samples will be collected less frequently (sparse sampling) to limit the number of timepoints and volume of blood collected.

Where feasible, 2-4 novel compounds will be administered simultaneously in a ‘cassette’ to reduce the overall 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?

All animals used will be from commercial breeders and where possible we will use a mix of males and female animals to reduce wastage at the supplier.

In parallel to the in in-life phase, we will work closely with our bioanalytical providers to reduce the volume of blood required for bioanalysis to allow the use of microsampling. This will have an impact both on total number of animals used and animal welfare (as small volumes of blood collection will be required).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be the first choice for studies but there are likely to be instances where this species is not suitable such as due to drug interactions or the way drugs are handled is not the same as in humans or where larger volume of blood might be necessary. In these cases rats will be used.

We will use the following to minimise harm to animals:

The most appropriate species will be used

Animals are kept in their social/cage mate groups.

Ensure that administration and sampling limits are adhered to.

Collect the minimum volume of blood on the fewest occasions

Use rigorous monitoring of clinical condition to ensure animals are euthanized within agreed severity bandings.

Continually assess published literature to ensure latest refinements are used and avoid duplicating work.

Why can’t you use animals that are less sentient?

Non-mammalian animals are limited in their use because they either do not have sufficiently complex bodies to reliably predict pharmacokinetics in higher species. For similar reasons, we are unable to use embryos or very young animals as the pharmacokinetics will not be predictive of adult animals.

Mammals need to be used for the following reasons:
The organisation of the biological systems in mammals for the metabolism and clearance of the drugs is highly differentiated from less sentient species.

Mice and rats have robust tails with good vasculature allowing multiple samples to be collected from a single animals with minimal stress.

The majority of efficacy studies using the best candidate molecules will be performed in mice or rats. Using the same species for the pharmacokinetic studies will provide accurate predictions of drug expose during therapeutic studies.

Animals need to be awake and mobile to accurately predict the pharmacokinetics in subsequent studies in efficacy studies in diseased animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be monitored closely for 2 hours at least post dosing, with another review at 4-6 hours post dosing then frequently observed following this (dependent on the duration of

the study). The humane endpoints in the licence will be closely followed to monitor potential adverse events such as abnormal/ laboured breathing, piloerection, changes in mobility to ensure consistent data records. If adverse events are observed the animals will be humanely killed.

Following each model, the clinical course of the animals will be discussed by the research team to ensure and lessons learned will be applied to future models.

To help in reducing the time animals are restrained and reduce the number of failed bleeds, the animals may be placed into a warming chamber to dilate peripheral blood vessels

As animals will only be briefly restrained over the course of a day, specific training prior to superficial vessel bleeds is unlikely to be beneficial.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

PREPARE and ARRIVE guidelines have been used from the outset of this program. The PREPARE and ARRIVE Guidelines Checklist have been completed prior to the application.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have an ongoing program of reviewing publications and attending conferences that address the 3Rs. We have also signed up for the NC3Rs newsletter and are in contact the NC3Rs program manager and attend regional, national and international 3Rs symposia.

50. Probing brain-body interactions in injury and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Neurological disease, Gut-brain axis, Neuropathology, Alzheimer's disease, Immunity

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	juvenile, adult, aged, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to identify and explore novel therapeutic strategies to treat neurological disease, as well as to explore the molecular milieu of the body and the brain that accompanies neuropathology. We are particularly interested in probing the communication between the brain and peripheral organs, such as the liver and the gut, and how the manipulation of this communication can alter disease outcomes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

With an ageing population in the UK, neurodegenerative diseases such as Alzheimer's disease and Parkinson's are becoming increasingly prevalent. Thus, it is vital that the most effective methods of research are used to better understand the pathology, risk factors, and potential therapeutic targets. Furthermore, it is currently estimated that over 450,000 people in England are living with severe disability as the result of a stroke, and each year an estimated 1 million people attend hospital A&E in the UK following a head injury. Of these, around 135,000 people are admitted to hospital each year as a consequence of brain injury, and the injury is often associated with long-term consequences including loss of function and personality changes. It is also worth noting that an estimated 2,500,000 people in the world have multiple sclerosis (MS) and there remains considerable pressure to better understand the aetiology of this disorder and provide earlier diagnosis. Our work will help scientists to better understand the cross-talk between the brain and immune system and it will benefit MS suffers and those that have suffered from an acute CNS injury by helping to identify new targets for therapy.

What outputs do you think you will see at the end of this project?

Our laboratory has a strong track record of disseminating and publishing new scientific information, which have been cited over 13,000 times, and it is expected that multiple peer-reviewed research articles will be published under the scope of this project licence. Through these articles, we expect to discover and disseminate new information regarding how non-cellular blood-borne particles allow for communication between the brain and peripheral organs (liver, gut, spleen) in health and in disease.

The work conducted under this license may also contribute to drug development in collaboration with industry partners. Under our previous licences we have managed to generate 10 patents, and some that have been licensed by a diagnostics company and others have led to the formation of spin-out companies.

Our research may contribute to new therapeutic strategies to diseases that cause a significant health and economic burden in the UK and internationally.

Our studies of diet and its effect on the host brain and behaviour may also inform future public policies around population health.

Who or what will benefit from these outputs, and how?

In the short to medium term the outputs are expected to benefit the scientific community both within and external to the establishment.

In the long term the outputs are expected to benefit the patient as it will help inform drug discovery and implementation of clinical trials. Our work on diet and its effect on the brain and behaviour may also inform public policies in terms of the health of the population.

How will you look to maximise the outputs of this work?

We will maximise the output from this study by publishing our work in open-access journals, presenting work at national and international scientific meetings, and making data

available in online repositories at the time of publication. We will also collaborate with other researchers to answer the project aims in the most effective manner possible.

Species and numbers of animals expected to be used

- Mice: 9100
- Rats: 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 are using mice and rats because they share many physiological and genetic similarities with humans that are relevant to the aims of this project. A large body of work over many decades has firmly established the laboratory mouse and rat as an excellent model of human diseases. Rodents have a short lifespan, allowing for maximum lifespan studies to proceed in a reasonable amount of time, and environmental factors that affect aging can be exquisitely controlled. Additionally, there are a variety of genetically modified strains available that can be utilised for neurodegenerative disease research, or to test the effects of therapeutic drugs. Finally, a large body of knowledge and techniques have been developed for studying the brain and behaviour of these animals, making it logical for us to design and carry out experiments in these species.

We will be using all life stages from neonatal animals through to aged animals of up to 24 months of age and including pregnant females.

Neonatal animals are important to show us the origins of chronic and neurogenerative disease in early development.

Pregnant females will be used to help us understand how the gut microbiome and the brain develop whilst the offspring are growing in the womb.

Aged animals will aid us in understanding the neurodegenerative diseases, such as Alzheimer’s, which are highly age dependent.

As we are looking to study diseases throughout the life span, we will utilize both juvenile and adult animals as well.

Typically, what will be done to an animal used in your project?

Genetically altered and germ free (lacking a microbiome) rodents may be bred, which is not expected to cause any harm. Pregnant females may be given modified food or a probiotic supplement to modify the maternal microbiome if we wish to study long term transgenerational effects of the changes. Other animals may receive modified diets, such as diet with increased fat content throughout their life to show effects of diet on disease.

Some (1-2%) animals will also be aged to 24 months to allow us to study those diseases that are dependent on ageing.

Some animals will undergo surgery to create a brain lesion that mimics that seen in the brain of patients with multiple sclerosis, or patients with a traumatic brain injury.

Substances that alter the function of the immune system, such as pro-inflammatory adjuvants and anti- inflammatory therapies, may be given by standard routes of injection, such as subcutaneous, intravenous, intraperitoneal, intramuscular. However on occasions we may inject substances directly into the brain via the intracranial route. These substances, if pro-inflammatory in nature, may cause a transient period of malaise, with symptoms like flu in the human, that is a result of a rise in body temperature. These symptoms are non-infectious and are self-limiting with a full recovery expected within 48 hours. Long term substance administration could result in a small slow delivery device being surgically implanted. Following surgery, we do not expect any harms that cannot be controlled with pain relief. An air pouch may be created by blowing air under the skin on the back of the animal to allow us to administer substances.

We will take blood samples from the tail vein to assess the levels of metabolites in an animal over time.

To be able to identify the effects of some of the diseases we study animals will be exposed to behavioural tests. These tests are non-invasive and do not cause any harm as the animal is able to choose to participate or not.

Some animals will undergo imaging via MRI under general anaesthesia to allow us to look at the injured brain as the animal ages.

What are the expected impacts and/or adverse effects for the animals during your project?

The use of imaging techniques reduces the overall number of animals required. Imaging allows for the non-invasive examination of the same animal over time (longitudinal studies). This reduces the need for large sample sizes where different groups of animals are used at each time point. Most of our models of human disease are clinically silent and we use these wherever possible. However, some animals might exhibit temporary weakness after the generation of a focal brain lesion, but otherwise we expect no clinical signs. Some animals (where the chronic inflammation is targeted to the gut) might develop diarrhoea.

Animals that are fed a high fat diet may undergo weight gain in comparison to animals on normal feed. We may also see insulin resistance, increased blood sugar levels and increased levels of inflammation none of which are expected to have any adverse effects. Age is by far the strongest risk factor in many neurological disorders including Alzheimer's disease and we may house some rodents that are up to 24 months old.

In extreme cases aged animals may suffer from seizures, weight loss/gain, degenerative joint disease that may cause mobility issues, dental disease such as overgrown incisors, palpable masses such as abscesses and benign tumours, cataracts, skin abnormalities such as localised patches of dermatitis, rectal prolapse and altered organ function. We do

not expect to see most of these symptoms in animals until they are at least 20 months of age.

Transient flu like symptoms such as reduced locomotion and hunched posture, will be seen in those animals that have had an inflammatory lesion created, however around 75% of these will be humanely killed within 24 hours and around 20% humanely killed within 3 hours if the injection has been directly to brain via the intracranial route.

In the case of germ-free mice (sterile mice lacking a microbiome) certain adverse effects may be expected. Specifically, germ-free conditions may lead to moderate problems with altered intestinal processes, leading to diarrhoea and dehydration (up to 5%). Mice may develop an enlarged caecum that is up five times that of conventionally housed mice. Torsion and obstruction of an enlarged caecum may occur and lead to death. In the experience of others who breed germ mice this is very rare (<3%) but is difficult to recognise. Mice will be inspected regularly for signs of an abnormally (for germ free) swollen abdomen and/or reduced mobility.

Some genetically altered rodents may have alterations that make them susceptible to neurodegenerative diseases. Some strains will show a higher level of spontaneous deaths due to epilepsy in their first year of life.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severity	Mice	Rats
Severe	0%	0%
Moderate	64%	99%
Mild	3%	0%
Sub-treshold	34%	1%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Many of the molecules that we know are important in the brain-immune system communication pathways are not expressed in fish or flies. It is important to note that in vitro work cannot model the complexities of the brain vasculature and the interaction with immune cells when designing and exploring novel treatment strategies that act within the brain. Our investigations typically involve a systemic approach to disease which demands a wholistic in vivo model.

Which non-animal alternatives did you consider for use in this project?

The substances we will study have already been screened or tested in vitro by us, using neuronal, astrocytic, macrophage, or endothelial cell lines. Where possible, we use ex vivo techniques, such as organotypic hippocampal slice cultures (OHSCs).

We considered other options to genetically modified animals, such as using in vitro cell-line models to relevant biochemical processes. While this approach may complement this project, it is not suitable as the main approach given that we aim to investigate the contribution of *systemic* immune activation and its effects on Alzheimer's Disease (AD) pathology. Furthermore, the blood-brain barrier is an important consideration when testing the therapeutic efficacy of novel drugs and drug targets, which cannot be effectively replicated in 2D cell cultures. In refining our experimental approaches, we also considered the use of 3D cerebral organoids to reproduce the rodent brain in vitro.

We have progressively made use of biobanked human data to explore our research questions. Employing this method can increases the relevance and translational utility of our work with rodents, and in some cases may replace or reduce the number of rodent experiments by making our hypotheses more targeted or clinically relevant.

Why were they not suitable?

Whilst the non-animal alternatives we have used complement our work they are not sufficient to answer all of the questions in our project.

Organoids are limited by a lack of cellular diversity, low synaptic activity, an incomplete immune system, and lack of a blood brain barrier. For example, we have previously observed in some of our disease models the recruitment of peripheral immune cells to the brain after the administration of immune-modifying compounds. This would not have been possible to discover in *in vitro* or *ex vivo* 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 expected number of animals is based on the Home Office returns data from the previous project licence, extrapolated to five years. Additionally, we recruited the assistance of a statistician to advise on the minimum group sizes for different types of experiments. Where behavioural experiments are required, we typically use 6-8 animals per group. Where we probe metabolism using metabolomic techniques, group sizes of 10-15 are needed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Prior to experimental design, a thorough and systematic review of the literature is performed to ensure that we are not unnecessarily reproducing the work of others using animals. Once the high-level design of the experiment is known, the NC3Rs experimental design assistant (EDA) was used to provide further advice on the most compatible statistical tests (e.g. parametric vs non-parametric, multiple comparison adjustments) to increase the robustness of the results and ensure appropriate reporting of statistics.

Most of the rodents we will use in this project are purchased directly from suppliers. Therefore it is extremely rare that we end up with surplus animals (in line with the PREPARE guidelines best practice).

When publishing data, we will ensure that the articles related to this project comply with the ARRIVE guidelines essential 10 components. as well as the recommended components of the guidelines.

We also practice reduction by the use of primary cell lines in experiments within the laboratory where we have a tissue culture facility. These cells may be derived from genetically altered or wild type mice and single animals can provide enough cells for many in vitro experiments. Repeated sampling and longitudinal imaging will be used to reduce the overall number of animals required. Before in vivo experiments are undertaken we hold a design workshop to plan the groups and group sizes to ensure that the experiment is appropriately powered (we will use the EDA for power calculations). The team also includes individuals with statistical training. It should be noted that we always make use of small pilots to ensure that animals are not wasted on large trials with negative outcomes. For each animal, both fresh and fixed tissue is usually collected to expand the scope for further experiments that will not require more animals. In this way, the benefit/harm ratio is increased.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

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 animals and to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced.

Tissue will be shared as much as possible, from us to collaborators, and vice versa, to reduce the number of animals used. For example, our collaborators maintain an extensive tissue bank for germ free mice. This has allowed us to conduct pilot studies using previously collected and stored tissue, without the need for additional animals.

The use of highly controllable experimental conditions in the behavioural neurosciences unit and optimized testing protocols can enable the reduction of total number of animals required for our behavioural experiments to be reduced by about thirty percent. The use of the local germ-free facility will allow for highly controlled conditions for experiments in germ-free mice, so we will likely use fewer mice when studying the microbiota than by other means e.g. antibiotic dosing of mice. We will also employ specialized video recording

in our open field and home cage monitoring experiments and tracking software to allow thirty-five social trait parameters be analysed offline. This dramatically increases the data return per animal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use germ free mice as opposed to giving long-term antibiotics to mice that already have gut flora as it causes less harm (antibiotics require a period of oral gavaging which is stressful for the animals). It is also the gold-standard approach for understanding cause-and-effect between the microbiota and whole systems of health, including immunity and brain development.

In vivo, genetically altered mouse and rat models of AD and other diseases capture important, translationally relevant aspects of the human disease, whilst allowing us to observe the influence of organs distal to the brain on disease progression.
To understand the pathology associated with inflammation of the central nervous system, some animals will undergo surgical procedures to elicit focal brain lesions. Our method is the most refined way to generate brain lesions, as the method of delivery does not cause additional brain injury or inflammation. This is because we usually use finely drawn microcapillaries for our injections, which also allows us to inject extremely small volumes that does not result in increased intracranial pressure.

Following the injection of a pro-inflammatory substances, the majority of animals (80%) exhibit no overt clinical signs, though "flu-like" sickness behaviour symptoms may occur in up to 20% of animals for
<48hrs.

In some cases aged mice will have to be used, typically up to 12 months, occasionally up to 24 months. This is the only suitable method by which to study age-dependent neurological diseases.

Why can’t you use animals that are less sentient?

Flies, fish, and worms do not possess the physiological complexity required to study neurological disease and the bidirectional relationships between the brain, liver, and gut. We cannot use terminally anaesthetised animals as we need to understand the effects of CNS injury/disease and therapies over longer periods of time and investigate effects on behaviour.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

BEHAVIOUR: Animals subjected to behavioural paradigms that are based on preference testing and do not cause any harm as the animal has an option to participate. The time spent in the testing arena is now only 5 minutes in duration.

AGEING: Ageing animals will be carefully monitored by staff trained to work with ageing animals. Group sizes in ageing experiments will be increased to accommodate for loss of animals and to avoid single housing due to animal losses due to old age. Longer drinking spouts will be used, and animals will be monitored for adverse effects such as changes in weight, dermatitis, piloerection, paleness, changes in mobility, lumps, eye defects, abnormal respiration, or stools. If these are observed animals will be treated accordingly, and animals that develop more advanced signs of ageing will be humanely killed.

SURGICAL PROCEDURES: Some discomfort is likely to accompany any surgical procedure. We will use analgesia for as long as necessary to alleviate this discomfort.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow LASA guidelines. The PREPARE guidelines and NC3Rs experimental design assistant will be used during the experimental design and action phases. The ARRIVE checklist will ensure we are complying with the best-practice guidelines for reporting data for publication.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our NC3Rs regional programme manager facilitates regular online or in-person seminars and workshops locally that will help advise on the replacement, reduction, and refinement of animals in this project. We attend the 3Rs research day and symposiums. Both myself and my students who are holders of a personal licence receive these emails.

51. Neural circuits for sensory perception and sensory-guided decision-making

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Brain, Neuronal circuits, Vision, Decision-making, Learning

Animal types	Life stages
Mice	adult, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The purpose of this project is to investigate how sensory information is processed by local and long range circuits in the brain and how this information is stored and converted into behavioural decisions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Given the essential role neurons, their connectivity patterns and dynamics play in brain function and dysfunction, developing an understanding of the processes that underlie neural computations and resultant behaviours is important in its own right. Our knowledge about the mechanisms of how the brain adapts, learns and uses information is even more limited. It is crucial to gain knowledge of how neural networks in the brain are organized and function under normal healthy conditions and identify the mechanisms that allow them to learn, since this is a prerequisite to understanding what goes wrong in the diseased

brain, for instance during neurological conditions such as schizophrenia or autism or during Alzheimer's disease.

What outputs do you think you will see at the end of this project?

Knowledge gained about the fundamental processes of how the brain processes and stores information and converts it into behaviour, which will be disseminated through peer-reviewed, open access publications in high profile journals and will be communicated through numerous scientific talks and conference presentations, as well as blogs and press releases for lay audiences.

This work will produce large amounts of physiological and behavioural data that we will make freely available.

Development of new tools to record and manipulate neuronal activity, for data acquisition and data analysis. All of these will be made available to the community.

The development of standardised hardware, software and protocols for behavioural experiments and physiological recordings of brain activity will lead to refinements of animal models and experiments.

Gaining knowledge of how neural networks in the brain are organized and function under normal healthy conditions is a prerequisite to understanding what goes wrong in the diseased brain and why, for example, cognitive processes and memory are impaired in Alzheimer's disease or psychiatric conditions. The identification of mechanisms that control information processing and plasticity in the brain may prove to be important for the development of novel treatments for correcting disorders of information processing such as autism or schizophrenia, hypo- or hyperactivity of circuits causing anxiety and depression, as well as disorders of information storage and retrieval associated with dementia.

Standardising experiments and identifying the sources of variability in animal experiments is likely to generate valuable insights for the general scientific community working on mice and might bring long-term improvements and refinements in the way mouse experiments are conducted.

Who or what will benefit from these outputs, and how?

The proposed project will provide fundamental scientific insights into the working of the brain. It will therefore be of significant interest to the neuroscience field in general, including systems, theoretical and cognitive researchers. Our work will produce foundational datasets that will drive forward our understanding of cognition and allow the field to make conceptual progress in understanding brain-wide information processing.

The scientific community will benefit from physiological and behavioural data made freely available, as well as access to tools for data recording, manipulation and analysis.

This work may have important implications for translational and clinical research, as it will provide a comprehensive basis of circuit function in the healthy brain that can be compared to brain function in mouse models of mental disorders as well as human patients with psychiatric conditions in which perception, learning or decision-making is

impaired. Our work could also contribute to identifying targets for therapeutic intervention for such conditions, including attention-deficit hyperactivity disorder or addiction.

How will you look to maximise the outputs of this work?

1.New knowledge gained by this research will be disseminated in international scientific publications and in scientific talks and presentations at national and international conferences and at various research institutions.

2.Data and Metadata, as well as software and analysis code will be made freely available to the scientific community to encourage further use of the gained knowledge and collaboration.

3.All technical advances will be made freely available to the scientific community.

4.Research outputs will be disseminated to the general public through press releases, blog posts and social media engagement and via public engagement talks and interviews.

Species and numbers of animals expected to be used

- Mice: 8300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 study will investigate how our brain processes sensory information, and how it uses this information and previously gained knowledge to make decisions and adapt behaviour to the environmental needs. These essential questions cannot be answered in the dish but necessitate studying the intact brain in a behaving animal. It is therefore impossible to avoid the use of animals for addressing these questions.

We will use mice in our studies. Mice are the species of choice in neuroscience and most areas of biomedical research, because of the vast library of existing genetically modified strains and other genetic tools, not available for other species. These tools allow targeting specific brain pathways and genetically identified populations of neurons, which is essential for dissecting the individual components of neural circuits participating in information processing and decision-making. Moreover, rodents are probably the phylogenetically lowest species for which direct comparisons can be made with the structure and functioning of the human brain. In addition, there is a vast amount of information on the neuronal physiology and synaptic transmission of mice, as well as on the anatomy of brain-wide circuits. Importantly, the visual system and decision-making networks in the brain are broadly similar between mice and humans, and therefore the results of this project will give fundamental insight into the function of the human brain without having to use higher order mammals, such as non-human primates.

We will use mostly adult mice to study the function of mature neural networks. In <5% of experiments we will use neonate/juvenile mice to compare their behavioural and neural

data with the adult stage or if expression of genetic constructs necessitates early intervention.

Typically, what will be done to an animal used in your project?

All experiments will be done using mice. In a typical experiment, a mouse will undergo surgery under deep anaesthesia with pain relief during and after surgery, to provide access to the brain and implant recording devices attached to the skull that enable neural activity to be measured in different parts of the brain. After recovery from surgery, the mouse may be trained to perform a behavioural task over several weeks up to several months depending on experimental requirements and task complexity, for instance to discriminate different sensory stimuli or associate a specific sensory stimulus with a reward. Mice may be food or water restricted to motivate their learning and task performance. Activity of brain cells in specific parts of the brain will be recorded in one or multiple recording sessions, in both trained or untrained mice. At the end of the experiment the animal will be killed humanely in order to collect brain tissue for histology and for collection of implanted devices.

What are the expected impacts and/or adverse effects for the animals during your project?

In preparation for the experimental procedures in this project, mice will undergo surgery under deep anaesthesia. Adverse effects after surgery may reach moderate severity levels for a short period of time because of post-operative pain, but all animals receive pain relief and are closely monitored until they recover completely, and usually recover fully after 24 to 48 hours. The majority of the procedures undertaken in this project involve studying voluntary behaviour in which the animals are expected to experience no or minor adverse effects. However, in some of the experimental steps the animals will be head-restrained in order to record brain activity and might initially experience stress from the head- restraint. But the mice will be allowed to slowly get used to the experimental conditions such that stress and discomfort will be minimized. Mice that are water or food restricted will experience weight loss, but these mice will be monitored and weighed daily to prevent adverse effects or excessive weight loss.
Recordings of neural activity will mostly be done non-invasively or with pre-implanted devices, causing no discomfort or adverse effects to the animal. At the end of experiments, or if mice show signs of ill health, distress or suffering that are not improved or resolved within a timeframe approved by the veterinary surgeon they will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most animals will undergo recovery surgery (> 95%), and animals with post-operative complications may reach moderate severity levels in some cases for a short period of time (< 24h). This surgery is essential for the experiment to allow access to the brain, implanting devices or expression of genetic indicators for targeting specific brain pathways and cell types.

Therefore > 95% of animals will be in the moderate severity category.

Few animals (< 5%) will only experience mild or no severity (either no surgery, or non-recovery anaesthesia).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project involves the study of dynamic properties of neurons, neuronal networks and their behavioural output in response to sensory stimuli. Studying live brain tissue is therefore essential for this fundamental biological research.

Which non-animal alternatives did you consider for use in this project?

Computer modelling and theory will be an integral part of this research work as it has in previous work to guide and constrain experiments to minimise the usage of animals.

We have also considered the use of brain slices and neuronal cultures.

Why were they not suitable?

As we learn more about the neural pathways and networks under investigation, we will be able to use mathematical modelling more extensively, but, for such approaches to be useful they will need to be tightly constrained with biological measurements. Work in brain slices and neuron cultures is not suitable for this project, as this work aims to understand how the brain processes sensory information and makes decisions. Therefore, the use of intact brains in behaving animals is unavoidable for the important scientific questions we would like to address.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 was estimated based on the experience of our group performing similar experiments and procedures over the last 15 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The experimental design of this study includes the employment of recent methods that maximise the amount of data collected from each animal. For example, with two-photon calcium imaging, we can record up to several hundred neurons at the same time, thereby reducing the number of animals required by at least 10-fold for key questions. The experimental design for most experiments also relies on using the same animals to gather experimental and control data under different conditions, as well as for ex-vivo analysis of the brain. This strongly reduces the number of animals needed, prevents duplication of experiments and reduces variability thereby increasing statistical sensitivity which again reduces the 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?

Whilst animals used in this licence will be obtained from other licences with the authority to breed and maintain GA animals, breeding strategy is also considered as part of this project to reduce the number of animals used. The breeding strategy for GA animals will be dependent on breeding performance of individual lines, litter sizes and demand. Where possible, lines will be maintained in a way that prevents generation of excess offspring with inappropriate genotypes, with regular review of experimental needs carried out and maintenance of breeding colonies that will supply this project.

The experimental design of this study also includes the use of techniques such as viral transfection which minimises the number of animals necessary for research, as it alleviates the need to make transgenic mice, which requires the breeding of many generations. Ordering in of animals for experiments instead of breeding will be considered if feasible, in particular for wild type animals. This will reduce the number of animals used, as animals will be ordered at the specific age or sex required, whereas breeding in-house may result in surplus animals being generated.

The statistical power of each experiment will be increased by measuring neuronal activity using different state-of-the-art methods that allow recordings of several hundred neurons at the same time, thereby reducing the number of animals required when compared with traditional methods. Refined statistical approaches and data analysis will be done in collaboration with computational scientists within the establishment, to extract the maximal amount of information from a particular experiment.

Computational modelling will also be an integral part of this research work to guide and constrain experiments to minimise the usage of animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Experiments will be limited to mice. We will use this animal model because phylogenetically it is the lowest species directly comparable to humans.

The proposed experiments are designed to enable maximum information extracted with as minimal pain and distress of the animals as possible. Throughout the project the main methods include stereotaxic surgeries, behavioural assays, electrical and optical recordings and micro-manipulation of neural activity. These methods have been continuously refined in my research group over the last 15 years. They are designed to enable maximum information extracted with as minimal pain and distress of the animals as possible. The large majority of recording and manipulation techniques are either non-invasive or performed with pre-implanted devices that do not cause stress or discomfort. For our experiments it is important that animals display their normal repertoire of behaviour, experiments in awake animals will therefore only be performed if the animals are stress-free and experience no visible discomfort. Also, manipulations of neural activity are genetically targeted to specific neural cell types, which minimises potential distress arising from global or off-target perturbations of brain activity. Other examples include the use of light-curable dental cement in all surgeries, which reduces surgery time by 20 minutes, and fibre optic implants often have magnetic connectors that can be attached in seconds without restraining the animal. We have also refined stereotaxic surgery procedures in general, including devising precise stereotaxic coordinates that minimise injection volumes and off-target effects. Surgical procedures will be done with appropriate anaesthesia and analgesia.

Why can’t you use animals that are less sentient?

Mice are the lowest species for which direct comparisons can be made with the structure and functioning of the human brain. Importantly, the visual system and decision-making networks in the brain are broadly similar between mice and humans, and therefore the results of this project will give fundamental insight into the function of the human brain without having to use higher mammals. This is not the case for less sentient species. Moreover, mice are the species of choice in neuroscience and most areas of biomedical research, because of the vast library of existing genetically modified strains and other genetic tools, not available for other species. These tools allow targeting specific brain pathways and genetically identified populations of neurons, which is essential for our study for dissecting the individual components of neural circuits participating in information processing and decision-making.

In addition, there is a vast amount of information on the neuron physiology, synaptic properties and neural circuit organisation of mice, which allows highly refined experimental design.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

When performing regulated procedures under anaesthesia we will ensure that the animals are sufficiently anaesthetised using standard procedures (e.g. pedal withdrawal, pinch reflexes, rate, depth and pattern of respiration).

We will use adequate analgesia and aseptic technique during any surgery and recovery periods to minimize pain that could be experienced by the animals due to surgical

manipulations. Analgesia with be given until full recovery. For procedures in awake head-fixed mice, great care will be taken to habituate the animals slowly to the experimental setup and the experimenter to ensure they are stress- free and in the majority of animals, neural activity measurements and manipulations will be performed with pre-implanted devices that cause no distress or lasting harm to the animals and enable normal behaviour.

Computational modelling will be used as part of this study, for help with designing experiments and maximizing the generation of statistically powerful data.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

- Refinements to rodent head fixation and fluid/food control for neuroscience. Barkus C et al., J Neurosci Methods. 2022. Nov 1; 381:109795

- LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. A report by the LASA Education, Training and Ethics section. (E Lilley and M. Berdoy eds.).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will keep up to date with 3R developments through the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs), including recommendations for best practices in neuroscience rodent experiments.

I will further be informed by the local 3Rs group at the establishment as well as animal facility staff and Named Persons, who I will work closely with to continuously implement 3Rs advances.

52. CRISPR/Cas9 library screening of gene mutation contributions to cancer development.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, CRISPR, gene editing, genomics

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?

Using mouse models that allow multiple gene edits to be introduced in parallel, we will investigate the relative disease contributions of a panel of cancer genes that contribute to 20% of all human cancers. We will investigate how tissue location of origin favours different combinations of mutations for driving and sustaining 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?

Many genes can contribute to cancer development, but some seem to be more important than others. Some of the most important are Ras genes that are mutated in 20% of all cancer patients. Moreover, the cell communication networks that Ras controls are mutated in almost all human cancers.

Treatments have been successfully created that reverse the effects of these mutations; however, cancers frequently become resistance to these treatments, meaning that further work is needed to extend the usefulness of these therapies. Ras consists of three closely related genes that can be mutated at various positions to promote cancer. We now understand that the Ras gene and mutation combination has a significant impact on whether cancer will occur. In order to study this and develop and test suitable therapies we need models of the Ras gene/mutant combinations. Our work will introduce a full panel of models and use these to study why different gene/mutant combinations are favoured in different tissues and how the cell communication systems respond to the mutants and in response to therapies. This represents an essential step for better understanding of the disease and the potential for personalised approaches to therapy. The models will be widely relevant to both discovery scientists and industry labs developing new Ras targetted therapies.

What outputs do you think you will see at the end of this project?

Primary outputs will be:

Publications - primarily addressing why some Ras variants are more oncogenic than others and the role of tissue context in determining this.

Resources - the Ras gene editing reagents will be made freely available via Addgene and the National Cancer Institute Ras Initiative. The mouse model will represent the first in vivo model where any of the 54 Ras mutants at codons 12, 13 and 61 can be tested alone or in combination.

Information - the know-how for efficiently introducing defined combinations of gene edits will be translatable to any other gene and therefore of relevance to any other group interested in systematically profiling panels of mutants in a single animal.

Who or what will benefit from these outputs, and how?

Academic benefit (short-term to medium-term)

The parallel measurement of the frequencies of a panel of mutations in lung cells and tumours will provide definitive data and publications about the relative oncogenicity of Ras isoforms and mutations – directly addressing questions that have existed for >35 years within the Ras field. The preliminary proof-of-principle work carried out by our collaborator already indicates the likely success of our study (Winters et al, 2017); importantly however, this previous work was not comprehensive and did not investigate the mechanisms underpinning the differences in Ras mutation frequency that they observed. We intend to profile the cancer cells with a panel of omic, imaging and cell signalling assays to understand the re-wiring that has occurred within the cancer cells, to identify the networks contributing to differences in oncogenicity and to understand the heterogeneity that exists both within and between tumours. Given the increasing appreciation of the role of tumour heterogeneity in driving resistance to therapies, our publications will be of broad relevance to cancer researchers and the approach will provide a platform for the efficient development of strategies to overcome drug resistance.

Translational impact (medium-term to long-term)

Nearly every major oncogene across every cancer type is associated with diverse oncogenic alleles. Despite growing interest in personalised medicine, there is only limited understanding of the functional differences (or lack thereof) among these clinical variants. Currently, the pharmaceutical industry rarely discriminates between different closely related mutations and isoforms when considering therapeutic strategies. Our publications on relative oncogenicity will demonstrate whether more attention needs to be given to these factors. The library screening approach that we are establishing provides a readily adaptable platform for pharmacogenomics analysis of lung tumours with different oncogenic mutations growing in a common in vivo environment – potentially allowing future translational studies to more accurately match the correct therapies to the correct patients. Finally, the omic and cell signalling analysis that we will perform have the potential to identify novel biomarkers and therapeutic targets.
This is important because attempts to directly drug Ras have largely failed and the most successful strategies have instead targeted pathways that Ras relies on to induce cancer.

Clinical impact (short-term to long-term)

Immediate impact will be seen upon publication of the data on relative oncogenicity of Ras isoforms and mutations. Currently, clinical trial design and interpretation does not discriminate between these variants due to the assumption that they are all equivalent – our data will either definitively confirm this assumption or provide a clear rationale for stratifying patients based on Ras variant status. Long-term impact may be seen if biomarkers, therapeutic targets or strategies for mitigating drug resistance are identified based on our work.

3Rs benefit (short-term to long-term)

The refinement and reduction benefits of being able to compare ≥40 different mutations in parallel within a single individual thus avoiding the confounding issues associated with inter-individual/inter- strain comparisons will be immediate for this project. They are also of relevance to any group using animal models to compare multiple or related genetic factors. As early adopters of this approach we have the opportunity to advocate the benefits to colleagues and to provide expert insight, relevant proof-of-concept data and confidence to other groups seeking to apply the approach to their research questions. This advocacy can begin once we have established the model and demonstrated successful gene editing. If widely adopted, the approach will significantly reduce the number of distinct animal models that need to be created and maintained, will increase the ability to confidently identify meaningful but subtle differences in gene variant biology being tested and will significantly increase the per-animal impact of any analysis that is performed.

How will you look to maximise the outputs of this work?

Our reagents will be shared without restrictions via international repositories (Addgene, National Cancer Institute Ras Initiative). We will be open to collaboration with any interested groups and will freely share knowledge and expertise. We publicise our work via national and international conferences, workshops, seminars and research publications. We have been presenting our preliminary data on the establishment of the Ras gene edited mouse model via posters and talks to engage potential collaborators in academia and industry at an early stage.

Species and numbers of animals expected to be used

- Mice: 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 mice because they replicate the genetic program that generates lung, colon and pancreatic cancer in humans. We will be introducing genetic mutations into target tissues in sub-adult and early adult mice (weeks 8-16) since it is known that the best gene editing efficiency is observed during this time frame. The gene edits will result in the development of an early stage of cancer in colon and pancreas 3 months later or in the development of small tumours in lung 6-9 months later. We need to wait for these visible signs of cancer to develop so that we can then investigate the co- operating factors leading to cancer development or to test inhibitors of cancer development.

Typically, what will be done to an animal used in your project?

1.Administration of a gene editing library delivered via a viral vector capable of infecting mouse cells. For lung via inhalation (intra-tracheal), for pancreas via tail vein injection, for colon via enema. Where appropriate, the procedure will be conducted under anaesthesia or sedation. All animals will be exposed to gene editing reagents.

The following procedures will be experienced by a subset of animals (likely <20%).

2.Adminstration of anti-cancer drugs via a superficial vein (eg. tail vein). Where appropriate, the procedure will be conducted under anaesthesia or sedation.

3.Blood may be withdrawn from a superficial vein (eg tail vein). Where appropriate, the procedure will be conducted under anaesthesia.

4.in vivo imaging using PET/CT scanning, ultrasound and/or fluorescence imaging to monitor lung tumour development. This may include the injection of contrast agents or fluorescent probes. To be conducted during pilot experiments to establish optimal time frame for tumour development whilst minimising harm. Where appropriate, the procedure will be conducted under anaesthesia or sedation or sedation.

5.Change to diet - animals may be given a variation in the composition of their diet for example to reduce any fluorescence or other signals that could interfere with pre-clincal imaging or delivery of gene editing reagents.

At the conclusion of the experiments the animals will be killed via humane methods.

What are the expected impacts and/or adverse effects for the animals during your project?

Administration of gene editing reagents - adverse effects are unlikely during the administration; however, any mice showing signs of problems breathing or toxicity (seizure, prolonged recovery from anaesthetic) shortly after dosing will be humanely culled

Withdrawal of blood - adverse effects are unlikely. To avoid anemia, blood withdrawal will be limited in volume and frequency.

Substances - the gene editing vectors have no reported toxicity, although a low grade immune reponse may be observed. We will be using some anti-cancer drugs with well understood toxicity profiles that we will monitor during the experiments. Doses will be no more than the maximum tolerated dose. The commonest side effects of the anti-cancer drugs are gastro-intestinal disturbance. Cytotoxic damages to the gut can result in a gastrointestinal syndrome that includes weight loss, diarrhoea, loss of electrolytes, and fluid. We will monitor for physical signs of ill health such as changes in posture, behaviour, nausea and mice exhibiting significant adverse effects will be immediate killed via humane methods.

A typical animal will develop multiple tumours of different sizes in the lung or multiple sites of pre- cancerous/early cancerous lesions in colon and pancreas. These are intended to be pre-symptomatic but sufficiently advanced to detect and use the disease material for subsequent analysis. For lung this will be 6-10 months post gene editing and up to 5 mm diameter for the largest tumours. For colon and pancreas this will be 3 months post gene editing and up to 5 mm diameter.

Pre-clinical imaging will be conducted to monitor lung tumour development in pilot experiments. Some imaging modes involve exposure to X-rays or gamma-rays that are potential harmful. Doses will be kept as low as possible and do not routinely result in any obvious side effects. Where animals will be repeat imaged over a period of months, we will ensure that the animal has recovered from previous anaesthesia ie. clinical condition including body weight have returned to pre-imaging levels.

The scientific needs of our experiments mean that we do not need to exceed moderate classification and will ensure that the constellation of any symptoms that an individual animal presents do not individually or cumulatively breach this. In the event that any animal exhibits signs of discomfort, ill health or weight loss approaching 20% of normal body weight at any point in the experiments we will consult with facilty staf and the NVS regarding whether these or any other symptoms are consistent with an irreversible trend towards a severe classification and if so they will be immediately killed via humane 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)?

None of the experiments will exceed moderate. In ≥70% of cases we expect mice to not develop clinical symptoms of disease. For most animals the main source of discomfort will be linked to the procedures for introducing the AAV gene editing vectors. For the pancreas and colon experiments (~20% of all planned experiments) the clinical endpoint will be colon hyperplasia or pancreatic intraepithelial neoplasia (early stage cancer) rather than

tumour masses. For lung experiments, the mice will be developing tumours, although our pilot experiments will define timecourses for minimising disease burden whilst still allowing us to obtain sufficient material for our scientific questions. The outcomes of these pilot experiments could then decrease our estimate of 80% reaching moderate suffering. The barcoding within our gene edits means that we measure differences in tumourigenesis via sequencing rather than needing to score many visible tumours ie. allowing us to address our scientific question at a much earlier stage of disease progression. This means that for all of our experiments except those where we need to allow visible tumours to develop and progress such as the drug response experiments (<10% of total) or where larger amounts of tissue are needed for mass- spectrometry based cell signalling analysis (<20% of total), we will be able to define disease endpoints that are likely to be pre-symptomatic. We think that the ~30% of total animals in experiments required for MS-based cell signalling/drug response analysis will be the main cohorts that could experience moderate suffering.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We have already performed extensive work in cell lines over the last 20 years to identify potential differences between Ras variants; however, it is not possible to extrapolate these in vitro differences into likely consequences for cancer development. Furthermore, we are aware of the influence that the

3D multi-cell type cancer microenvironment has on inter- and intra-tumour heterogeneity and this cannot currently be replicated using in vitro systems. For our data to be of clinical relevance, it is important that these factors are present in our experiments.

Which non-animal alternatives did you consider for use in this project?

We are specifically interested in oncogene/tumour suppressor gene variant contributions to lung, pancreas and colon cancer development. Therefore, the zebrafish melanoma model that has been applied to Ras mutant oncogenesis analysis and the C. elegans egg laying model used for Ras pathway activity assays are not appropriate. Human data already tell us that Ras variant-specific behaviour is likely to be observed; however, the lack of availability of fresh patient samples suitable for use in the variety of post-in vivo analysis pipelines that we employ means that we cannot generate detailed mechanistic insight. Furthermore, the variability that exists between patients presents significant confounding challenges for data analysis that are not present in our mouse model where all mutations are compared in a single experimental unit.

Why were they not suitable?

Our research questions are aimed at trying to understand the interplay between different Ras mutants and tissue context. We know from the work that established the model in

2017 that there are significant differences in the cancer causing potential of different Ras mutants and that their potency varies between tissues. In order to understand the reasons for this we need access to Ras mutated tissues to be able to perform the mechanistic analysis that we are expert in. We have specifically chosen the three main cancer types where Ras mutations are most frequently observed. Zebrafish and C.elegans do not currently have Ras driven models for these cancer types. Furthermore, these non-regulated species have ancestral Ras genes that are not the same as in humans. Therefore, the inability to replicate the contextual signalling details and the specific cancer types that we are interested in means that there are not suitable alternatives. Human patient samples will be used to corroborate our main findings; however, they are unsuitable for mechanistic studies because they cannot be experimentally manipulated over time to investigate the consequences of different Ras mutants in the same genetic background.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Advice on the proposed experimental designs and methods of analysis of the results has been taken from the retained statistical advisor for our animal facility. Our experiments are examples of factorial experimental design where up to 20 variables are being compared with each other within a single

experimental unit. In total we will be using this format to investigate 54 different Ras variants, this means that we have reduced by >50-fold the number of different mouse models and associated breeding programmes required to achieve our scientific objectives. Based on support from our statitistical advisor and data from previous publications we expect that 9 animals per biological replicate should be sufficient to quantify significant differences in oncogenicity of a large panel of Ras variants based on genomic analysis. However, due to the wide range of ex vivo analyses that we intend to also perform, it may be necessary to use more animals per group to obtain sufficient sample yield for these other qualitative and quantitative studies; this will be assessed in the in vivo pilot experiments that we will perform prior to any large scale experiments. For estimation purposes we have suggested 8 additional animals per biological replicate but will iteratively refine this number to use fewer animals for ex vivo analysis if possible. The ex vivo approaches further maximise the information obtained per animal. Where possible, these approaches will be integrated so that the results obtained by multiple analyses of an individual sample will directly inform the interpretation and conclusions of each other.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

An important feature of these experiments is that all mutations are compared in a single animal. This means that instead of having a different mouse colony for each Ras mutation we are studying, we have a single mouse colony where any of our panel of Ras mutants

can be studied individually or in combination. The cells are exposed to each CRISPR vector at random and only one mutation will be integrated per cell meaning that there will be no confounding effects of multiple variables integrating into each cell. By comparing all of these mutations in a single animal we are able to control for the inter-individual variability and inter-treatment variability that are common confounding factors and therefore the approach should provide greater sensitivity to observe biological differences and have confidence in the conclusions drawn.

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 that we will use will be obtained from a breeding colony that will be maintained under the Project Licence of our animal facility. Efficient colony management ensures that the colony only produces animals when actively being used and colonies that are no longer required are cryopreserved and closed at the earliest opportunity. The facility has extensive experience and success of predicting the number of matings required to generate the experimental cohorts specified over time in experimental plans.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 represent the preferred pre-clinical model within the Ras pathway drug development field. The same genes that are known to contribute to human lung, pancreas and colon cancer drive lung tumour development in mice and a range of Ras driven models have already been developed. Current approaches rely on the generation of independent conditional mutant mouse models. The Ras work described in our proposal would have required the generation of up to 54 Ras mutated mouse lines. Generating a new genetically modified mouse allele including the cloning, embryonic stem cell targeting, blastocyst injections and subsequent breeding requires between 1.5 and 3 years before in vivo experiments are even initiated. The time and resource required for generating mouse models mean that only 12 out of 54 potential Ras models are currently available. In contrast, our approach allows any combinations of these Ras mutant alleles to be compared in parallel in a fraction of the time (<18 months to generate reagents and to perform an initial round of experiments) and maximizes the scientific impact per mouse.

Our approach relies on the introduction of gene editing reagents to induce Ras-driven lung, colon or pancreatic cancer. The use of quantitative genomics to precisely quantify cell numbers with each mutant allele – rather than relying on counts and analysis of large tumours - means that even subtle differences in oncogenicity can be identified. Confidence in these comparative differences is increased because they are being compared in the same individual where most potential confounding factors are removed. This means that

the biology that we are interested in can be studied at a lower level of disease burden and reduced harm to the individual.

Why can't you use animals that are less sentient?

Our work relies on the development of visible tumours that take 3-12 months to grow following the introduction of the gene-edits. We will also be exploring responses to drugs that target the Ras pathway. There are no alternative less sentient species with suitable Ras-driven disease models that would be compatible with these aims. The long time course of the experiments precludes interventions that decrease the sentience of the mice that we will be using.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Both our library CRISPR animal model and our quantitative genomics approach represent significant refinements in efficiency and sensitivity on current standard practice. We also intend to refine the exposure of the animals to potential harm and suffering through pilot experiments that aim to reliably identify the earliest time points for collecting sufficient types and volumes of samples that are consistent with the scientific aims. For the scientific aims of the project it is necessary for visible tumours to develop rather than simply in vivo survival and proliferation of gene-edited cells. However, the techniques that we are using are sensitive and do not require high disease burden for the generation of sufficient material. During the pilot phases of the project we will determine the optimal balance between disease development and sample yield for our planned analysis – aiming to define humane endpoints that keep to a minimum the time and burden of disease experienced by the animals

in the subsequent programmes of experiments whilst also minimizing the total number of animals required for the experiments.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For experimental design and reporting the Experimental Design Assistant, and ARRIVE guidelines are relevant. The model that we are employing was published in 2017 and the group responsible for this have since published several high impact research articles that have firmly established the state-of- the-art. We are collaborating with this group. Their lab is 100% focussed on developing mouse models and new tools that enable cancer biology to be systematically profiled. We continue to collaborate to ensure that we have advanced access to any refinements that the group that developed the model identify.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our animal facilty and the Named Veterinary Surgeon regularly circulate information relevant to latest guidance and policy. The Head of Home Office Facilities at our University is establishing an NC3Rs group that will specifically focus on 3Rs activity, raising awareness and linking with external initiatives and funding opportunities. We will continue to interact with the NC3Rs gateway, to ensure that our plans and priorities remain current and compatible with national advances.

53. Carotid chemoreflex activity and cardiovascular risk

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cardiovascular, autonomic, hypoxia, chemoreceptors, respiratory

Animal types	Life stages
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project will investigate the mechanisms that make carotid bodies hyperactive following repeated falls in blood oxygen levels (called intermittent hypoxia) as seen in sleep apnea patients and how changes in the associated reflex responses contribute to the increased cardiovascular risk that is also present in sleep apnea 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?

The prevalence of sleep apnea is increasing globally and is often linked to the global increase in obesity (a risk factor for developing it). Sleep apnea patients are also at much greater risk of developing cardiovascular diseases such as hypertension (high blood pressure) and atrial fibrillation (a disturbed rhythm and function of the heart). Current treatments are often focused on treating the outcomes (eg lowering blood pressure by drug treatment) rather than addressing the driving force behind the cardiovascular disease.

The carotid bodies are sensors located in the neck that detect many circulating factors including, importantly, oxygen levels in the blood. In sleep apnea, there are multiple periods of intermittent hypoxia that over time drive changes (called remodelling) in the carotid bodies making them hyperactive even during normal oxygen levels. Understanding the mechanisms underlying the changes in the carotid bodies will help deliver targeted treatments to counter the hyperactivity.

Stimulation of the carotid bodies evokes normal reflex responses that in addition to changing breathing (respiratory responses) also change autonomic nervous activity and this impacts on cardiovascular function. An example of this normal reflex response is the increase in breathing you experience when going up a mountain to altitude. This reflex pathway starting from the carotid bodies, resulting in both cardiovascular and respiratory changes, is called the peripheral chemoreflex. Changes in this peripheral chemoreflex pathway are likely to be contributing to the increased cardiovascular risk. As well as generating a reflex response, hypoxia can also have a direct local effect on tissues. In the carotid bodies this is what stimulates them, however, a direct effect of hypoxia on the heart can change cardiovascular function and result in increased cardiovascular risk. It is important to be able to distinguish between these effects to understand the mechanisms behind the increased risk.

Understanding the changes in carotid body function and the impact this has on reflex autonomic nerve function may provide a novel therapeutic approach for treating cardiovascular risk in these patients.
Modulating carotid body function may provide a therapeutic target at source rather than just addressing symptoms.

What outputs do you think you will see at the end of this project?

This project will provide new knowledge on the potential mechanisms underlying the higher risk of cardiovascular disorders in obstructive sleep apnea (OSA) patients. The new information generated by this research programme will be communicated to the scientific community and the general public through presentation at national and international conferences and publishing in journals. It is hoped that the results will open up new therapeutic targets to help treat these patients whilst preserving their basic reflex response.

Who or what will benefit from these outputs, and how?

In the short-term, the impact of these outputs will be to inform the scientific research community through publishing in peer-reviewed journals. The outputs will lead to a greater understanding of the role of the peripheral chemoreflex in driving cardiovascular risk under these conditions.

In the medium-term, since this project will give a greater understanding of the role carotid bodies may play in driving cardiovascular risk, it is hoped to ultimately inform a new therapeutic approach that treats the cause rather than symptoms in patients. This research would be able to provide a proof of concept for this approach.

In the long term, it is hoped that modulation of carotid body activity will provide a discrete target to counter it's hyperactivity in different diseases whilst retaining basic reflex functionality.

How will you look to maximise the outputs of this work?

As the main output from this basic research will be to publish peer-reviewed papers, the outputs will be maximised by publishing in high quality journals. Negative data will also be published to ensure scientific colleagues do not need to carry out the same experiments and use more animals than necessary.

Species and numbers of animals expected to be used

- Rats: 600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In order to investigate the complex adaptive and reflex responses induced in the cardiovascular and respiratory systems by chronic disease, it requires a mammalian species with sufficiently similar physiology to that of humans. Even in humans, the carotid bodies are only the size of a grain of rice and so the animal species chosen must provide enough tissue to actually experiment with. Additionally, the in vivo approaches require a species that is large enough to record all the cardiovascular and respiratory variables reliably.

Zebrafish are often used to consider genetic and developmental aspects of both cardiovascular and respiratory research, however, there is no published research where the integrated nature of cardiorespiratory responses (ie both together) has been successfully conducted in zebrafish as part of understanding the chemoreflex response.

Mice have a chemoreflex where both cardiovascular and respiratory outputs can be measured to some degree, however, are not directly applicable to all our experimental approaches. For example, the carotid sinus nerve that arises from the carotid bodies to send information to the brain is too small in mice to record from directly. Researchers have attempted to overcome this limitation by recording from the petrosal ganglion - but this means functional additional identification of a nerve in the petrosal ganglion arising from the carotid body before any experiment can begin. This "hit and miss" approach is less refined than ours (where we are able to record from the rat carotid sinus nerve directly) and so would likely require more animals to acquire data.

The rat is the species of choice for this research as its physiology is very similar to humans and we also have a track record of successfully achieving good experimental results from this species both in vitro and in vivo. We are able to successfully record nerve activity from the carotid sinus nerve, work on isolated hearts, as well as making all of the cardiorespiratory measurements in the whole animal. The mechanisms involved in carotid body function, reflex responses to carotid body stimulation and autonomic control of the cardiovascular system are very similar to humans.

The life stage to be used is adult, and is usually a young adult so that chronic adaptation can be studied without the confounding factor of aging in addition. The chemoreflex matures/resets to the adult phenotype within a couple of weeks after birth meaning young adults are the earliest age that can be used with adult responses and sufficient tissue available. A recent estimation of OSA global prevalence (from 16 countries) suggested nearly 1 billion adults between 30-69 years old had moderate to severe OSA and so the condition is not limited to old age.

Typically, what will be done to an animal used in your project?

Approximately half of the animals will only undergo terminal non-recovery procedures. For these studies, stock animals will be terminally anaesthetised (ie without recovery) and tissues harvested for lab investigations or, prior to killing, cardiorespiratory measurements will be made. Tissue harvesting is conducted under deep terminal anaesthesia to minimise the periods of hypoxia (low oxygen) and metabolic insult suffered by tissues known to be oxygen-sensitive as this would interfere with the scientific purpose. The studies are completed by a Schedule 1 method of humane killing.

Approximately half of the animals will have their blood pressure or ECG taken non-invasively to give a baseline reading before proceeding into the remainder of the experiments where blood pressure or ECG may also be non-invasively monitored further.

The majority of these animals will undergo periods of intermittent hypoxia (exposure to a minimum of 5% oxygen for 10-15 seconds before rising back to 21% oxygen again, a maximum of 30 cycles an hour for up to 8hrs a day). This will be repeated over a maximum of 4 weeks so as to induce chronic adaptation of the carotid body and drive subsequent reflex changes, modelling that experienced in sleep apnea.

The number of cycles per day and number of weeks exposure may be reduced as well as the minimum oxygen levels being higher, if a moderate sleep apnea is being modelled.

Some of these animals will be administered a drug chronically via a previously implanted pump to investigate the ability to modulate the chronic adaptation of the carotid body and changes in reflex responses and so understand the underlying mechanisms. Some animals may receive a single injection prior to non-invasive cardiorespiratory measurements being made after CIH exposure.

Measurements of acute respiratory responses may be made in conscious animals at the end of the chronic intermittent hypoxia period to investigate the changes in carotid body-induced respiratory reflex responses. This is done using Whole Body Plethysmography (WBP) where an individual conscious freely moving animal is placed in a chamber (2.7L in volume) and exposed to altered respiratory gases (typically 10% O2 for 5 min and 6% CO2 for 5 min). As the animal is free to move (measurements can only be made when the animal is settled), these recordings may take up to 2 hours.

In some animals non-invasive blood pressure and ECG measurements may be made up to twice a week to track the chronic adaptation to intermittent hypoxia and, when chronic drug administration is used, to investigate the effect of the drugs on the chronic adaptation. These recordings involves temporary restraint of the animal in a tube (typically under 15 min)

At the end of the experiment, animals will be placed under terminal anaesthesia (ie without recovery) where additional invasive cardiorespiratory measurements may be made to provide more detailed data and/or tissues harvested for subsequent ex vivo experiments.

What are the expected impacts and/or adverse effects for the animals during your project?

Sometimes animals will need to be restrained for non-invasive measurements (eg ECG or blood pressure) and so be exposed to mild stress during this period. Restraint is achieved by allowing the animal to go into a tube with the exit closed and the animal will often sit there quietly without the exits closed. These are temporary short-lived procedures and animals undergo minimal stress usually.
Animals that do not acclimatise or tolerate the restraint tubes will be withdrawn from the procedure.

Exposure to chronic intermittent hypoxia is expected to induce short-lived changes in behaviour initially. The fall in O2 will induce visible increases in breathing (as a result of stimulating the chemoreflex response) and this is often associated with a temporary reduction in physical activity at the outset (reducing O2 consumption). On return to higher/normal levels of O2 then normal behaviour returns and as animals adapt these behavioural changes are less obvious. Animals that are left undisturbed during these periods (during daytime) will often sleep and so the increases in breathing are the only observable effects. The animals are unaware of the adaptive changes happening and these can only be observed during experimental measurements.

Drugs may be administered over prolonged periods (to give chronic dosing) by prior mini-pump implantation. This will involve surgery to implant a device under the skin that can release the drug slowly. Animals are expected to recover quickly from the implantation and will be given peri-operative analgesia and post-operative care.

Plethysmography recordings involve placing the animal into a recording chamber (of 2.7L volume), however, they are completely unrestrained and able to freely move inside the chamber. To help reduce the stress of an unusual environment some home cage bedding is also transferred with the animal.
Assessment of the chemoreflex will be done by varying the gases flowing through the chamber (hypoxia and/or hypercapnia), the reflex responses include changes in breathing pattern and for hypoxia a temporary reduction in activity (to conserve oxygen) – as the animal is conscious it is aware of the change in its breathing pattern. These reflex responses are quickly reversed with the return to normal air breathing.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 50% of the animals used will be for terminal non-recovery procedures.

Approximately 50% of the animals used will have an expected moderate severity with the cumulative effects of intermittent hypoxia, chronic drug administration or non-invasive recordings on conscious 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?

This project focuses on a reflex pathway originating in the carotid bodies (located in the neck) that is integrated in the brainstem and then, following differing levels of cardiorespiratory interactions in the brainstem, produces respiratory responses (changes in breathing) and cardiovascular responses (such as heart rate and blood pressure) via changes in autonomic activity. These reflex responses are in addition to the direct effects of hypoxia that can be observed in isolated tissues. These reflex responses cannot be studied in any in vitro system alone as the complexity cannot be replicated.

Which non-animal alternatives did you consider for use in this project?

We have used, and will continue to use, in vitro cell culture based approaches that may give some insight into the mechanisms involved in individual cell types to hypoxic responses. These approaches are used in combination with animal studies so as to inform the project direction and reduce the number of animals needed for subsequent studies wherever possible (for example, we use cell-based studies to screen potential pharmacological interventions so only the most promising are taken forward into animal studies).

This project licence is centred on integrated reflex responses that require a multitude of cell types, located in different areas of the body. The sensors (carotid bodies) are made up of different cell types (primarily type 1 and type 2 cells) that induce nerve activity that inputs into the brainstem (an area of integration). The brainstem then drives different outputs (cardiovascular and respiratory targets) as part of the reflex response that affects function in different tissues (such as heart muscle and respiratory muscles). The complexity of this integrative physiological response means it cannot be studied fully in non-animal alternatives.

Why were they not suitable?

Whilst we do have cell lines that are oxygen-sensitive, and so can be used to study some of the mechanisms involved in the hypoxic responses of the carotid bodies, they are derived from tumour cells and are not "normal" cells. The ideal in vitro model would be based upon carotid body specific cells, but unfortunately this does not currently exist and there are still no commercially available carotid body specific cell lines due to the difficulty in producing them with appropriate characteristics.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated numbers required to provide a robust answer to any set of experiments will be calculated using information gained from a mix of:

1. the published literature using comparable techniques

2. our own published data using the techniques

3. pilot experiments (especially if effective drug concentrations are not published for a similar use)

Tissues taken at the end of animal experiments for either subsequent in vitro experiments and/or further processing (eg histological analysis) will reduce the total number of animals used to produce the required results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

At the experimental design stage the minimum group size required to confidently interpret the data will be calculated as described above. For example, this means a group size will differ between in vitro tissue experiments and in vivo experiments due to the sources of variability within the different systems.

In order to reduce the variability (and so the number of animals used), all experiments will use standardised protocols to generate the primary data. This will include using animals of comparable ages (to reduce any age-related variability) for specific experimental approaches and acclimatising animals before any protocols using conscious animals to reduce the anxiety/stress in the animals during measurements.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To keep the number of animals used to the minimum, where possible, tissues will be taken after in vivo experiments and used for further studies and/or analysis. In vitro experiments may be possible in some circumstance (eg if no drug has been administered), however, wider use of tissues for further analysis (eg histology) will be possible.

We will also look to collaborate with other researchers to provide tissue for their research from tissues that would not be used within our objectives and would otherwise just be disposed of. We will continue to keep abreast of the literature so that no animal experiments will be conducted when there is good published evidence already.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Approximately 50% of the animals used will only experience terminal (non-recovery) anaesthesia. These animals will only be aware of the induction of terminal general anaesthesia. Approximately 50% of the animals used will be exposed to procedures that influence adaptation of the chemoreflex pathway but are not expected to produce overt visible changes to the animals.

The physiological pathway being investigated in this licence is a reflex pathway that undergoes adjustment/remodelling in the experimental models, however, no outwardly observable phenotype is associated with exposure to intermittent hypoxia (because, for example, hypertension is not "seen"). When animals are exposed to CIH, the whole home cage (complete with cage mates) is placed inside the hypoxic chamber. The familiar scents and cage mates reduce any anxiety caused by transferring into a different environment.

In order to explore the mechanisms involved in the adaptation of this reflex pathway, chronic drug administration will be required. The use of osmotic pumps to deliver the drug of interest over an extended period will reduce the need for repeated handling and injections. Analgesics will be used peri-operatively to further reduce any pain or distress to the animal induced by the surgery. Overall these approaches will reduce the overall level of distress any animal will experience during the experimental protocol.

Where measurements are made in conscious animals (eg breathing measurements), the animals will be exposed to the recording environment in advance in order to acclimatise them. We have also found that transplanting some home cage bedding (familiar scent) into the plethysmography chambers also allows the animals to settle more quickly. Measurements made on conscious animals are all short- lasting and induce minimal stress with the animals returned to home cages as soon as possible. These steps reduce the anxiety of being in a new environment during measurements and so help reduce variability leading also to a reduction in numbers as well as reducing anxiety in the animals.

Why can’t you use animals that are less sentient?

This research project investigates the mechanisms underlying the generation of reflex responses and then how these are modulated/changed to increase risk of death in humans. Animals that possess a similar reflex and function in a similar way to humans are required to investigate this. Rodents possess the lowest level of complexity and sentience for the results to be interpreted meaningfully. Whilst some of the investigations into the reflex mechanisms use component parts (eg carotid body or heart), the intact animal is required to measure the overall reflex responses and rats are the smallest rodents where it is feasible to make all the measurements required concurrently.

Approximately half of the experiments are conducted on isolated tissues or terminally anaesthetised animals. The other half of experiments involve the generation of a sleep apnea model (chronic intermittent hypoxia) before non-recovery anaesthesia to make cardiorespiratory measurements and/or harvest tissues which may also involve chronic drug administration.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In experiments where measurements are made on conscious animals, the animals will be familiarised to the environments on occasions prior to the measurements being made. This training will reduce the stress of the animals during measurements as well as giving more reliable measurements.

In experiments where there is chronic drug administration via implanted pumps, pain management will be employed pre- and post-implantation with the advice of the NVS/NACWO being taken. The use of these pumps reduces the need for repeated handling and injection of drugs over extended time periods.

When animals are put into the environmental chambers they are observed at the start in order to ensure they do not show any unexpected adverse responses to the protocol.

When new protocols or drug regimes are introduced, a pilot study will be conducted in order to ensure there are no adverse effects prior to using the drug in a larger group of animals. We do not foresee any requirement to use new drugs that have not been previously characterised and so would not require pilot studies to be undertaken for this purpose.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The planning of the experiments will be conducted using the PREPARE guidelines to ensure they are planned and conducted in the most refined manner.

During the experiments, LASA guidelines will be followed for the routes and volumes of drug administrations.

After the experiments, the data will be written and published in accordance with the ARRIVE 2.0 guidelines for reporting animal experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive the monthly NC3Rs newsletters via email and these provide information on latest advances.

In keeping abreast of the published literature in this field, we will also monitor for new advances that may be incorporated into our approaches.

New advances from any source will be discussed and assessments made on how they may be integrated into the way the experiments are conducted

54. Investigating the genetic basis of salmonid resistance to sea lice

Project duration

1 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Sea lice, Atlantic and coho salmon, Disease resistance, Improved welfare, Sustainable food production

Animal types	Life stages
Salmon (Salmo salar)	juvenile, adult
coho salmon (Oncorhynchus kisutch)	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to investigate the genetic basis of resistance in salmon to parasitism by sea lice. The project will identify genes and mutations affecting resistance to support the development of sea lice resistance in farmed Atlantic salmon.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Food from water is a critical element to satisfy the protein requirements of a growing global population. As returns from fishing for wild fish in open waters has largely remained constant since 1990 aquatic food production is moving from wild capture to sustainable farming. Aquaculture's contribution to the supply of fish for human consumption exceeded that of wild-caught fish for the first time in 2014. To meet the continually growing global demand, it is estimated that aquaculture production will need to further double in the period to 2050.

Despite progress aquaculture still faces many problems. Infectious diseases of viral, bacterial, protozoan, and parasitic origins are the most significant constraint, causing lost production, increased costs, wasted resources, major animal welfare problems and concern for transmission to wild stocks. Atlantic salmon aquaculture is worth approximately £1 billion per annum to the UK economy (at first sale) and is a major source of employment in rural communities of the Scottish Highlands. While the Scottish Government and the aquaculture industry has ambitious plans for sustainable growth, infectious diseases constrain this expansion.

Sea lice (particularly Lepeoptheirus salmonis in the Northern Hemisphere and Caligus rogercresseyi in the Southern Hemisphere) are a significant, perpetual problem for salmon aquaculture globally. These copepod parasites attach to the skin of salmonid fish and feed on mucus, live tissue, and blood.

Parasitised fish show impaired growth and increased occurrence of secondary infections. Furthermore, potential transmission of sea lice to endangered wild salmonids is a key issue motivating regulators to adopt more precautionary policies to aquaculture, constraining development. In addition to a significant negative impact on salmonid health and welfare and public perception, lice prevention and treatment cost the global industry about £800 million per year.

A diversity of control strategies including area management plans, reducing the duration of the marine phase of salmon production, feed supplements, cleaner fish, mechanical removal (by water jet, brush, laser), bathing treatments (freshwater, high temperature), and tailored cage design exist or are being developed. These multifaceted strategies are only partially effective and can cause additional welfare issues. Veterinary medicines, which are expensive and potentially environmentally damaging, are still frequently required to control sea lice, however resistance to common delousing drugs and adverse welfare during treatments are constant concerns. Therefore, despite extensive control efforts, sea lice remain a major hindrance to sustainable aquaculture, including negative effects on public opinion of the industry.

A wide diversity in response to infectious disease is a well-known phenomenon both between, and within species. Some host species such as Atlantic salmon are highly susceptible to parasitisation, while others (e.g., coho salmon) are highly resistant. Improving the innate genetic resistance of aquacultured salmon to lice is a promising, environmental and welfare friendly, yet underexploited approach to control.

Traditional controlled breeding programs for key production species have enabled enhancement of key traits, including disease resistance over the long term. In the last two decades advances in genome sequencing allowing cost effective individual and family typing have enabled breeding programs to enhance and speed up this process by using

marker assisted selection. The identification of a marker associated with resistance to infectious pancreatic necrosis virus (IPNV) in salmon and its incorporation into breeding programs is a good example of this resulting in major reduction in incidences of IPN disease globally and associated massive welfare improvement. Understanding the mechanisms and genes underpinning the observed variation in resistance or susceptibility to diseases is now key to enabling and implementing more specific and even more rapid future control strategies based on selective breeding.

Future selective breeding programs based on gene associated selection need to be informed by small scale empirical studies. Studies such as those proposed within this licence have the potential to support a sustainable increased production of seafood with immense welfare improvements (due to the large numbers of individual animals farmed) and reduced chemical use and environmental impacts.

What outputs do you think you will see at the end of this project?

The immediate outputs will include fundamental knowledge on the mechanisms of genetic resistance to sea lice parasitism which will be disseminated via high impact publications and international symposia. Important outcomes also include future targets for selective breeding programs and novel strategies to combat sea lice impacting on aquaculture, so industry focused publications, meetings and conferences will also be targets for dissemination.

Who or what will benefit from these outputs, and how?

In the short-term the data will improve understanding of the mechanisms behind increased host resistance to sea lice and academia will benefit from empirical data driven publications. In the short to medium term the aquaculture industry will benefit from knowledge on future relevant targets to include in traditional selective breeding programs enhancing resistance to sea lice in Atlantic salmon.

Beyond the duration of this licence in the medium to long term the outputs benefit rural economies engaged in aquaculture at the national and international levels offering reduced production losses, improved public perception of aquaculture, food security, employment, and associated benefits.

Previous work on identifying markers associated with resistance to infectious pancreatic necrosis virus in Atlantic salmon (now used in traditional selective breeding strategies) is estimated to have a value of approximately £26 million per annum to the UK industry alone. As breeding programs are introduced for new targets identified under this licence and become effective there will be further massive welfare improvements for both future farmed fish experiencing reduced impact of disease and added benefit for wild salmonids by reducing parasitism pressure from aquaculture.

How will you look to maximise the outputs of this work?

The work is collaborative across academic and industry partners, as such dissemination will be wide and active via all partners in the project. High impact publications as well as informative workshops and solutions for industry to take forward into breeding programs will be disseminated.

Species and numbers of animals expected to be used

- Salmon (Salmo salar): 1000
- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The fish used are the appropriate species for the investigation, i.e., the relevant farmed species and natural host (Atlantic salmon) and a naturally resistant but non-native, non-farmed species (Pacific, coho salmon).

Coho salmon have been brought into the research facility as eyed eggs under special dispensation from stock enhancement programs in Canada. Juvenile Atlantic salmon of defined family makeup with predicted differing susceptibility will be provided by industry partners. Both species will be held as stock animals and reared until the post-smolt seawater stage when lice exposure will occur. We will also be using juvenile stages of Lepeoptheirus salmonis parasitic sea lice obtained from an experimental aquarium facility on the Atlantic coast of Scotland. Copepodid lice are crustacea.

Decapod crustacea (class Malacostraca) are now legally recognised as sentient in the UK and elsewhere. Although sea lice are a related class (Copepoda) within the superclass multicrustacea, and may meet some of the criteria around awareness, they are not yet recognised as sentient.

Typically, what will be done to an animal used in your project?

Lice eggs strings will be collected from parasitised salmon, transported to the experimental aquaria held in static aquaria with regular seawater exchange, hatched to infectious copepodid stage, enumerated and added to tanks containing salmon in seawater.

Both species of fish in seawater will be exposed to lice copepodids in controlled disease challenges by immersion followed by sampling at defined timepoints. As it is known that the differential resistance manifests (by rejection of lice) in the first few days after attachment challenges will be held for a short period only (7 days maximum). Fish will be terminated humanely one tank of each species at a time, across up to nine timepoints by a Schedule 1 or an approved non-Schedule 1 method. Fish will experience a single exposure procedure.

What are the expected impacts and/or adverse effects for the animals during your project?

Initial settlement of copepodids can elicit flashing and jumping, indicating irritation, but behaviour usually returns to normal within 1 week. Sea lice challenges will usually be conducted by lowering the water level and adding infective copepodids. Following parasitism with lice, most fish show slight skin damage. This is most often on the dorsal midline, immediately posterior to the dorsal fin and is characterised by a white/grey opacity and thickening of the epidermis with occasional pinpoint haemorrhages. In heavier infestations, lice may induce more extensive and marked damaged to the epidermis, characterised by exposure of the dark pink underlying dermis. This is normally only after

the lice have developed to mobile pre-adult and adult stages. In such cases this would be categorised as acute moderate harm and the fish would be killed immediately using a schedule 1 method. This study is short term and will complete well before lice reach the pre-adult stage.

The actual adverse effects are managed by defined humane endpoints implemented by regular appropriate monitoring which involves both direct visual checks and desktop observations of live videos from in-tank underwater cameras. Actual severity experienced will be mitigated by timely removal and humane termination if necessary.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity is mild and the maximum severity is moderate.

As this experiment will assess host response to lice attachment in the early stages after attachment, up to a maximum of 7 days, the lice will not develop into mobile pre-adults or adults which are the stages that are most damaging to the fish and when epidermal damage and dermal exposure might be observed. The most likely severity fish will experience is mild, transient irritation.

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 attachment site of the salmon skin comprises multiple tissues and cell types, and differential resistance may be due to actions of specific cell populations. Susceptibility and response to parasitism thus require complex interrelated metabolic, anatomical and immunological mechanisms that cannot currently be fully adequately modelled except in a protected whole animal.

During the project we will also use a small number of salmon to establish in vitro primary cell cultures from skin to investigate and corroborate potential target genes identified in the comparative disease challenge and subsequent host response analysis. This will become a future resource that will be used in future experiments under a different project licence using gene editing to test empirically the impact of these gene targets. However, data derived from in vitro studies are not typically entirely accurately reflective of the whole animal response.

The fish used are the appropriate species for the investigation, i.e. the relevant farmed species and natural host (Atlantic salmon) and a naturally resistant but non-native, non-farmed species (Pacific, coho salmon).

Which non-animal alternatives did you consider for use in this project?

Computer based (in silico) models and cell culture based (in vitro) models will be used in the project to inform choice of gene targets for future investigation. Analysis of production data, genomic data from earlier experiments, and mining of existing data from online databases as well as the transcriptome data from the current project will be used to inform candidate resistance genes and mutations. High priority targets identified from transcriptome analysis of the comparative host responses will be tested in cell culture models: this will include genome editing of target loci in the cells, enrichment for edited cells and exposure to parasite secreted protein. Future projects under a different licence envisage empirical testing of target resistance genes by genome editing of salmon embryos (in vivo). Measures of cell survival in the cultures developed in this project will inform the likelihood of a gene edit having an effect on resistance in future in vivo experiments. These cell cultures, of which there are relatively few compared to terrestrial animal and human models, are necessarily derived from relevant fish species.

Why were they not suitable?

Cell cultures derived from humans or terrestrial animals are not appropriate for fish disease research as in most cases the pathogens of cold blooded fish do not replicate at the temperatures terrestrial animal cell culture models are held. The immune system of invertebrates differs considerably from that of fish, preventing direct comparisons in pathogen challenge with invertebrate models. Fish cell culture models are informative to a certain degree but are not solely sufficient since infection barriers and the immune system have many different elements and span various organs and tissues. Thus, whole animal models remain essential to this research area.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated numbers based on number of experimental units, accounting for replication, husbandry requirements (particularly stocking density) for both species and contingency to ensure sufficient fish at the seawater stage for disease challenge.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We and associated project partners utilise professional statisticians, the statistical software R and, when relevant, packages for power analysis to ensure animal experiments have adequate statistical power to detect at least medium sized effects in the assessment of lice attachment and comparative host parasite expression analysis. We refer to acknowledged tools for best practice such as PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) and fish specific guidelines (NORECOPA) and the Experimental Design Assistant (EDA) for design of experiments, replication will be used, allocation to tanks will be randomised, and sample analysis will be

blinded to increase robustness and repeatability ensuring resilience. We use ARRIVE guidelines for eventual reporting. This approach is intended maximise likelihood of a successful challenge experiment providing appropriate results and data and minimise requirement for experiments to be repeated.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All animal research conducted at the laboratory is undertaken using approaches consistent with defined Good Laboratory Practice regulations. The high-quality standards provide assurance that very few studies need verification or repeat. The number of animals required for each study is calculated in conjunction with our Statistical Services Group. This ensures that the minimum number of fish are used to provide robust data. If sufficient baseline data is available the use of control animals may not be required, to reduce animal usage. Stocking densities and population size remain a necessary consideration to ensure expression of normal feeding and social (e.g. schooling) behaviour and to minimise anti-social (aggressive) behaviour. We continually review and improve infrastructure in the experimental facility to enable further reduction of animal numbers within experiments. Examples include additional sizes of tank and systems to achieve variable depth control in tanks allowing reduced numbers to remain at appropriate stocking densities thus fewer fish required for studies. A detailed formal study plan is prepared for each study undertaken, and contains study specific objectives and justifications for use, severity of expected adverse effects and the number of fish required. Study plans are reviewed by the local Animal Welfare and Ethical Review Body (AWERB), fish husbandry experts and the Statistical Services Group, ensuring that all the 3Rs have been considered and animal use is reduced to the minimum required to achieve the aims of the study.

The use of effectively specific pathogen free fish by rearing from eggs in the bio-secure experimental facility, means numbers for negative controls can be kept to an effective minimum. Project partners have longstanding expertise in lice challenge models. Tissues from sacrificed fish will be shared across multiple partners in the wider research program, including linked projects not directly funded by the grant awarded for this work. A small pre-study using a small number of fish from each group will be performed to confirm effective transfer of lice challenge skills between project partner establishments and inform lice numbers for the main challenge.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 model to be used is parasite challenge by immersion in water. Salmon are raised according to each species preference across each life stage, the specifications of which are obtained from review of relevant literature and expert opinion and detailed in species

specific husbandry cards. Features include (not exhaustive) temperature, salinity, pH, dissolved oxygen, excretory product levels, hygiene, light intensity and photoperiod, stocking density, nutrient requirements, recommended feed types, growth rates, and environmental enrichment (e.g., water movement).

Salmon in seawater will be exposed to sea lice by addition of a defined numbers of copepodids per fish into replicate experimental tanks containing up to 45 post smolts. These will be maintained for up to a maximum of 7 days post exposure. Copepodid challenges will be performed on predicted high resistance Atlantic salmon, predicted low resistance Atlantic salmon, and resistant coho salmon.

Demonstrated consistency in fish holding conditions across study groups and appropriate replicates by precise control of environmental parameters will ensure repeatability of the challenges with minimal variation.

Skin and attached live parasites will be sampled from fish in each group per time point, with counts of attached lice and fin clip samples taken from all fish. The lice count data will be used to benchmark the resistance phenotype of the Atlantic salmon families and inform on precisely when lice are rejected by resistant groups of fish. Atlantic and coho salmon post-smolts will be kept separate under appropriate husbandry conditions. Each tank will have a predefined sampling timepoint for termination to avoid repeat disturbance of fish during the challenge. Because the study focuses on the early time post attachment and holding is for a short period only, the lice will not mature to pre-adult or adult stages and thus the potential epidermal damage and harm experienced by the fish will be limited.

Why can’t you use animals that are less sentient?

Sea lice naturally exist in marine waters and parasitise the marine grow out stage of the salmon life cycle. Sea lice do not survive in freshwater hence the early life stages of salmon are not appropriate. The study is an in-depth analysis of host parasite interaction in the first days after attachment. It is not pragmatic or sufficiently representative to maintain large numbers of fish in many tanks under anaesthesia for this duration.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Routine monitoring for salmonids is three times daily whilst feed is offered manually. This provides the ability to check the fish as well as assess feeding behaviours ensuring all fish get equal access.

Checking will be increased immediately post exposure to lice and throughout the study if signs of epidermal damage are observed. Each individual is checked against agreed criteria and observation recorded on score sheets. Visual checks will be supported by additional video surveillance.

Because behavioural changes associated with irritation such as flashing and jumping are expected transiently after attachment of lice, tanks will be netted and will contain minimal furniture to reduce possibility of physical damage to fish as a result of this behaviour.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We utilise the PREPARE and fish specific guidelines (NORECOPA) and the Experimental Design Assistant for experimental design. We use ASPA codes of practice, Guidelines for the use of Fishes in Research (American Fisheries Society), Guidelines on the care and use of fish in research, teaching and testing (Canadian Council on Animal Care), and any relevant news articles from NORECOPA, LASA, AWRN, and IAT.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The project is short term for one study only; opportunity to implement changes during the life of the project are therefore limited. However, in the lead up to the study the project licence holder is part of the local AWERB which facilitates continual professional development via named information and training officers and regular surveillance and dissemination of new 3Rs literature. The licence holder will also benefit from access to the principal investigator and project licence holder of project partners who have extensive experience of maintaining lice affected salmon populations and running lice challenges.

55. Assessment and treatment of donor organs for transplant

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

Transplant, Therapy, Repair, Machine Perfusion

Animal types	Life stages
Pigs	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?

During the process of organ donation, organs undergo a period of injury before they are removed and they then need to be preserved and transported to the transplant centre. This results in differing amounts of damage to the organs and means that some organs are turned down for transplant.

We want to develop ways to treat, test and repair donor organs so that more organs can be used for transplant and more patients have an improvement in their quality of 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?

More than 400 donor kidneys and 50% of all donor pancreases removed for the purpose of transplant are not transplanted each year in the UK. This is because doctors are uncertain of the quality of the organs and at the moment there are no accurate ways of testing whether organs are suitable for transplant or not.

There is a plan to develop specialist centres where donor organs are sent for testing and repair. At these centres, new techniques such as machine perfusion, where the organ is pumped with a warmed oxygenated solution, are carried out. Machine perfusion offers the opportunity to assess the organs and deliver therapies to try and repair them.

There is also a clinical trial currently running that delivers high dose statins to organ donors before the organ donation operation begins. The objective is to try and reduce the injury to organs before they are removed. This study has set a helpful precedent and there are many other therapies in development that could be given in this way.

What outputs do you think you will see at the end of this project?

We are developing novel methods to assess, maintain and improve donor organs so that fewer organs will be declined based on concerns about their suitability for transplant. This will lead to more organs being transplanted and improved quality of life for patients.

We anticipate that the outcomes from this work will be used to inform Normothermic Machine Perfusion protocols and interventions that can be tested in the clinical setting through the establishment of specialist organ assessment centres.

Once complete, we will submit the outcomes of this work for presentation to relevant regional, national and international societies. We will submit the results for publication in a high-impact peer-reviewed journal. Where possible, results and outcomes will be disseminated to patient groups and charities.

Who or what will benefit from these outputs, and how?

The ultimate objective of this research is to increase the number of donor organs transplanted so that patients benefit by removing the need for dialysis and improving quality of life. The translation of this form of research from the laboratory to into clinical practice takes time and we anticipate that patients will start to benefit in the next 5-10 years.

How will you look to maximise the outputs of this work?

There are several research groups and industry partners in the UK who are interested in this area of study and with whom we currently collaborate. We will ensure that positive and negative results are disseminated and published so that advances continue to be made irrespective of research outcomes.

Species and numbers of animals expected to be used

- Pigs: 90

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Juvenile pigs are an ideal model for donor organ research as they have very similar anatomy, physiology, biochemistry and immunology to humans. They are a similar weight to an average human adult and are therefore more manageable from a veterinary and surgical perspective compared with young adult and older pigs. Their abdominal and thoracic organs are a similar size to adult human organs and they therefore are ideal for translational research.

Typically, what will be done to an animal used in your project?

Pigs will be anaesthetised and mechanically ventilated during the procedure by either intubation (as in human surgery) or via a tracheostomy. Depth of anaesthesia will be monitored by conventional methods as used in human surgeries. Some blood vessels will be cannulated during the procedure so that we can administer substances such as heparin to prevent blood clotting and some therapeutic substances to support the organs that we wish to harvest. Periodically during the procedure, we will take blood samples to assess the gaseous values of the blood and the metabolic status of the animal. It will also be used to monitor the anaesthesia regimen. We will be using the application of probes to monitor the organ of interest (typically the kidney and pancreas) and the blood vessel parameters.

In some pigs, we will perfuse the animals with organ preservation solution to aid our in vitro studies. Once we have harvested our organs and any other tissue that we require the animal will be humanely killed whilst still under anaesthesia.

The expected duration of the procedure is expected to be around 4 hours.

What are the expected impacts and/or adverse effects for the animals during your project?

There are no expected impacts or adverse effects expected during this project as all procedures are performed under terminal anaesthesia, which is administered and maintained by qualified personnel.

Expected severity categories and the 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% non-recovery

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Declined human donor kidneys are available for research, however they are very varied in their characteristics which makes comparison between organs difficult and means that it is not possible to use them for very precise experiments. They are also offered for research when they become available, which may be overnight or at the weekend which means it is not possible to plan experiments and important resources may not be available.

Pig organs are very similar to human organs and are an ideal substitute. They are accessible from slaughterhouses and specialist farms and are an important step in showing the potential of therapies and interventions prior to translation to human studies.

Which non-animal alternatives did you consider for use in this project?

The use of human kidneys that have been declined for transplant can be used for research. This is a resource that we have used in the past and continue to use for certain studies. These kidneys are very variable and are useful in the very late stages of research, immediately before moving into clinical practice but are less useful in early stage research.

Why were they not suitable?

As, mentioned above, there is large variation in human kidneys declined for transplant. The differences include, very long preservation times, organ damaged during retrieval, blood not flushed adequately and the presence of cancer. This means that research questions requiring precision cannot be answers with this resource.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We estimate that each series of experiments will require about 6 animals. The maximum number of groups per year is estimated at 3 and over 5 years that means about 90 animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use a combination of slaughterhouse and farm animals bred for research. The

slaughterhouse model utilises organs and blood from animals undergoing slaughter for meat that would otherwise be wasted. This resource is excellent for very early stage research but there is large variation between animals due to the mode of death which means they are of limited value in research assessing subtle changes. By incorporating slaughterhouse animals and declined human kidneys into the experimental design we are able to minimise the number of farm-bred animals we use for research.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We try and utilise as many organs from each animal as possible by combining different studies so that kidneys, liver and/or pancreas can be used from one animal in three separate projects. In addition, we used paired designs for our kidney experiments which strengthens the experimental design and means that half the number of animals can be used, as one kidney can undergo treatment and the other kidney acts as the control.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 juvenile pigs as they are the most suitable model to replicate the human organs. By harvesting organs under terminal anaesthesia, we do not anticipate any pain, suffering, distress, or lasting harm to the pig other than the mild stress it experiences by being in a laboratory environment shortly prior to anaesthesia.

Why can’t you use animals that are less sentient?

The pig is the least sentient of the mammals with adult organs similar to humans in anatomy and size that is available.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All of the procedures will be performed under general anaesthesia which includes sedation prior to any intervention and pain relief will be given throughout surgery. All procedures are under terminal anaesthesia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I am a practising clinician and remain up to date with advances in clinical techniques. I will ensure that through contacts with the named Veterinary Surgeon and lead Named Animal

Care and Welfare Officer I remain up to date with any changes in surgical protocols to improve refinement.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The establishment provides regular updates and information via email and on the 3Rs website which ensures that animal licence holders are well informed. I use these resources to keep up to date and would alter the protocols accordingly if any advances meant protocols or interventions were updated. We also work very closely with the Named Veterinary Surgeon and lead Named Animal Care and Welfare Officer to ensure that any advances in the 3Rs are observed.

56. Development and repair of cranial structures

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

Embryo, Jaw, Head, Repair, Genetic

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?

To make precisely engineered animal models to understand cranial development, and explain the underlying causes of congenital defects that impact the head.

To use knowledge of development to enhance repair and regeneration of cranial structures in later 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?

In our lab, we investigate how the craniofacial region develops during embryonic and early postnatal life and how his knowledge can be used to enhance repair and regeneration in

later life. Both normal and abnormal development are studied to understand how the tissues and organs of the head interact to form such a complex structure. Worldwide approximately 3-6% of babies each year are born with a serious birth defect. Of these, craniofacial abnormalities (involving the complex tissues of the head) are the most common and collectively represent about one third of all congenital anomalies. While some of the underlying genetic causes have been identified, we still lack an understanding of the mechanisms that link mutations in specific genes to the physical anomalies associated with most craniofacial birth defects. These clinical conditions have life-long consequences for the individuals affected, their families and the community they live in. To achieve a beneficial outcome in the health management of craniofacial disorders, a major goal must therefore be to develop effective strategies for early diagnosis and prevention of craniofacial disorders and to improve the efficacy of treatment. Ultimately, this requires in-depth understanding of the pathology and etiology of craniofacial malformations, and of the underlying genetic, environmental and developmental causes.

What outputs do you think you will see at the end of this project?

Results from this study will advance our knowledge of the underlying causes of human developmental disorders including the formation of clefts, jaw defects, dental defects, ear defects, and reveal how defects in one part of the face can impact other regions. Knowledge of how these organs form in the embryo will also inform how to bioengineer and enhance repair during adult life.

We will be able to link genetic changes to cellular changes to understand why mutations in particular genes cause cranial defects. This knowledge is important for developing new therapeutics and providing families with a better understanding of anomalies.

Our major outputs will include new information for the public, patients, clinicians, and researchers. This will take the form of peer-reviewed publications and presentations and public engagement activities.

We will also generate new mouse models and data to be shared with the scientific community. Outputs from this work will include:

Peer-reviewed scientific publications.

Scientific datasets deposited in public repositories. Lay summaries for use by Charities.

Presentations at conferences Collaborations nationally and internationally
Engagement with relevant patient organisations

Public engagement activities at festivals and museums

Who or what will benefit from these outputs, and how?

In the short term our outputs will add to knowledge of the fundamentals of human and animal craniofacial development and provide knowledge of repair mechanisms for cranial tissue. The outputs will inform the bioscience and clinical communities of key findings of relevance to human and animal health.

In the long term, this information will lead to new innovations in diagnosis, prognosis and therapeutics, and new repair/regeneration strategies for craniofacial tissues.

How will you look to maximise the outputs of this work?

We will aim to maximise the outputs of this work by:

- the publication of findings in open-access journals collaborating with international teams
- encouraging knowledge exchange by presenting at specialist and public events working with patient organisations and the general public to share our new knowledge
- Sharing our resources with the scientific community by using publically available repositories Hosting visiting students and researchers to share expertise

Species and numbers of animals expected to be used

- Mice: 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.

Advances in transgenic mouse technology makes the mouse a powerful system for understanding craniofacial biology. The latest technology allows for introduction of genetic changes at precise times in precise tissues to allow an understanding of the role of genes at a level not previous imaginable. We can track populations of cells throughout life, and switch genes on and off to understand their function over time. In this project we investigate genetic changes throughout an animal's lifespan (embryo to adult). Most of our work focused on developing stages when tissues are forming (in the embryo and first weeks of life), but we also investigate adult mice to understand how the same tissues undergo repair after damage. As mammals, the development of many cranial organs is similar in mice and humans, with a number of transgenic mouse models mimicking human craniofacial syndromes (Treacher Collins syndrome, 22q11 syndrome, Branchio-oto-renal syndrome, Down syndrome, EvC syndrome etc) allowing us to ask clinically relevant questions.

Typically, what will be done to an animal used in your project?

For the majority of the project, we are using genetically manipulated mice for breeding purposes. Male and female mice are placed together for natural mating, which should cause no adverse effects and is classed as mild severity (protocol 1). These embryos and pups are used to model human disease and are carefully monitored and culled if signs of distress are shown.

To trigger some genetic changes, or for cell labelling purposes, we will administer compounds that alter development or allow us to follow the fate of cells. Such agents may be given in food or water, or by injection. Administration of compounds is also routine and should cause no adverse effects. Mice will experience mild, transient pain and no lasting

harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal) (Protocol 2). For collection of embryonic tissue, pregnant mice are culled using Home office agreed procedures (Schedule 1). Most offspring will need to be genotyped, in order to understand their genetic makeup. This is typically performed by taking tissue samples, such as ear notching.

To understand and enhance repair in adult mice, damage will be induced using standard injury models of craniofacial tissue (protocol 3). In all cases, damage will be minimal and occur under anaesthetic with pain relief provided.

After all procedures, animals will be monitored for discomfort and will be killed by a Schedule 1 method if discomfort becomes evident. Humane endpoints will be determined for each protocol. In general most animals are culled for analysis once they have reached 12 weeks of age, although breeders (particularly male mice) will be housed for longer (Protocol 1). Animals undergoing surgery are usually culled 1 week-3 months after surgery, unless they are being used for a longer term lineage experiment (Protocol 3).

What are the expected impacts and/or adverse effects for the animals during your project?

While some of the genetic modifications will have no obvious effect, such as those used to follow cells during normal development, others may produce tissue changes, such as cleft palates in embryos, which would cause perinatal lethality. In these cases, all embryos/pups would be collected before or at birth, before the onset of difficulties. We expect that many of the developmental abnormalities will be observed in the embryo, and, therefore, will be collected as embryonic tissue. Induction of genetic changes during postnatal development may impact feeding and sensory development. All genetically engineered animals will be monitored closely to mitigate any unanticipated welfare concerns. Newly engineered lines will be monitored particularly closely as it is sometimes difficult to anticipate the effects of specific genetic modifications on individual animals. For animals with any jaw, minor palate, or dental issues we will feed a gel or mash diet to alleviate potential pain, and animals will be monitored for any weight loss. Such soft diets would be given for a few months and long-term use would not be the norm. Animals will be monitored for discomfort and will be killed by a Schedule 1 method if discomfort becomes evident.

Expected severity categories and the 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 the majority of our animals will be used for breeding and generation of embryos and neonates. We expect minimal impacts, with 10% mild and 90% sub-threshold.

We anticipate that the establishment of new genetically modified lines will lead to some unanticipated phenotypes, which will need to be carefully monitored. For these lines animals are culled prior to overt signs of distress.

Mice will be culled prior to developing any issues which would be considered moderate. For example, EVC knockout pups, model the human syndrome Ellis Van Creveld, and are

generally of small size with enamel defects. These mice are culled before they reach one month old, which is before the onset of other issues (such as heart defects).

Damage procedures will be performed on adult and juvenile mice and will have a 100% moderate severity as they involve recoverable surgery after damage, and animals are likely to experience short term moderate pain or distress.

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?

Animal models provide a great deal of information on the causes and progression of developmental cranial defects. In particular, animals with the potential for genetic manipulation are necessary for studying the molecular events underlying human development and repair. These kinds of studies need to be performed in vertebrates where analogous genes are implicated in the development, healing, and regeneration of analogous structures. Many cranial structures studied in the lab are unique to mammals, these include the jaw, dentition and ear. For example, the dentary bone forms the entire lower jaw bone in mammals but is only one bone in a composite lower jaw in non-mammals. The mammalian middle ear transfers vibrations using three bones, while the non-mammalian ear uses just one. Mammalian teeth occlude and sit in fibrous sockets, while non-mammalian teeth are generally attached to the bone and interdigitate. These differences mean that it is essential to use a mammal to understand human cranial defects and repair strategies.

Importantly, development and repair involve tissue interactions in a 3D structure requiring the need for in vivo analysis to understand how cells interact over time to create complex 3D shapes.

Which non-animal alternatives did you consider for use in this project?

We have pioneered the use of explant and slice culture techniques to culture murine tissue ex-vivo and use these routinely in the group. This has been utilised successfully to study tooth development, salivary gland development and early stages of ear development. We are also working to improve computational models of development, which can help us reduce and refine our experiments as well as to narrow down hypotheses.

Why were they not suitable?

Cell culture is currently not able to provide the complex 3D environment of multiple tissue types in order to recreate the development of intricate organs, such as the ear. Although very useful, our explant culture approaches can only replicate simple tissue interactions

and are restricted by the length of time tissues can be cultured for. The cranial region is created from interactions between endoderm, ectoderm, neural crest and mesoderm in complex geometries, with dynamic cell movements and waves of signalling over time. It is just not possible to mimic this complexity in non- animal tissue.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Total numbers of animals needed for breeding and experimentation have been estimated based on prior experience with these protocols. The majority of mice are for breeding with mild or sub-threshold severity.

Although we will not perform quantitative tests on our live mice, we will on the tissue collected from the animals that we are using under this licence.

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.
Where appropriate, we will use the EDA diagram and report outputs to support experimental planning with animal users.

For the majority of our experiments, we will be able to use littermate (sibling) controls in order to minimise variability between animals, using inbred strains such as C57/bl6. Samples sizes for our experiments will be estimated from past experiments, and using power calculations, with minimal numbers of animals used whilst ensuring that results are statistically significant.

For repair experiments, we can use the contralateral side of the head as a paired control, allowing us to reduce sample sizes.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will share tissues with our project partners in order to maximise research application. We have ongoing collaborations with researchers interested in brain and body tissues and store unused transgenic tissue generated by our experiments for use by other researchers.

Importantly, we study a range of tissues in the cranial region (ears, jaw, teeth, glands, palate) that all interact during development, or share an evolutionary history. Therefore, multiple questions can be answered from analysis of a single mouse.

All researchers in the lab are trained in efficient breeding practices to make sure crosses generate the correct genotypes.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In order to understand cranial development, we need a genetically tractable mammalian system that can mimic the human life cycle. In this project we use mouse, which as a mammal develops its cranial structures in a similar manner to humans. The mouse remains the most powerful system for studying mammalian genetics. We will use mouse breeding, mouse injection, oral administration, and injury models.

Breeding of transgenic mice allows us to follow specific populations of cells in the embryo and postnatally, and to remove or enhance the expression of specific genes at key timepoints, or restricted to specific tissues. These are very powerful tools for understanding the role of genes in development and repair. The breeding is classed as mild severity, as any transgenic mice generated that could show more moderate phenotypes will be culled before they reach this stage (for example as embryos, or young neonates). In order to trigger the genetic changes, or modulate gene and protein expression, it is necessary to administer biological reagents. These will usually be via an IP injection (causing minimal distress and pain) but the method of delivery will be dependent on the reagent in question, for example some reagents require oral gavage. Where possible we will use the method that cause minimal pain and distress. The injury models selected are relatively minor (caused by needle insertion) and are classed as moderate. In protocol 3 we are using a needle to make a small hole in the ear drum (tympanic membrane) under recoverable anaesthetic. Such holes heal normally within 2 weeks and cause minimal pain. We will not use any major surgery. The injury methods are, therefore, designed to cause minimum stress and pain on recovery.

Why can’t you use animals that are less sentient?

The group uses a range of animal models to answer questions about development. These include chick and reptile embryos. However, many cranial structures studied in the lab are unique to mammals, these include the jaw, dentition, palate, and ear. For example, the dentary bone forms the entire lower jaw bone in mammals but is only one bone in a composite lower jaw in non-mammals. The mammalian middle ear transfers vibrations using three bones, while the non-mammalian ear uses just one.

Mammalian teeth occlude and sit in fibrous sockets, while non-mammalian teeth are generally attached to the bone and interdigitate. These differences are linked to the evolution of a unique feeding mechanisms in mammals and mean that it is essential to use a mammal to understand mammalian cranial defects and repair strategies. However, the majority of the research investigates development so uses immature forms.

For our injury models, mice are required to recover from minor surgery so we can track the repair process, so non-recoverable surgery is not possible.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals are monitored after any procedure to check for discomfort. After minor surgery pain medication is delivered and mice are not placed back in their rooms until they have fully recovered from any anaesthesia. During surgery and recovery, mice are placed on heated mats to ensure they do not lose body heat and handling is minimised.

Suffering is minimised by placing the animals in as stress-free an environment as possible – with spacious cages with plenty of spaces to hide, no overcrowding, and housing with suitable companions.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We regularly review and update current best practice. We will follow published guidelines such as the PREPARE guidelines.

We consult current published references include:

Animals in Science Regulation Unit (ASRU) Guidance and regulatory advice, such as:

Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for ScientificPurposes Guidance on Breeding Protocols for Genetically Modified Mice Guidance from LASA www.lasa.co.uk

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 have signed up to the NC3Rs newsletter, we will meet the NC3Rs Regional Programme Manager, and attend Regional 3Rs symposia. We participate in national and international meetings where group share best practice on genetic engineering approaches. This keeps us informed on current advances in animal welfare and research and will allow us to efficiently share any improvements that we make in our project.

57. Genetic and functional changes in congenital anomalies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Developmental biology, Embryology, Congenital anomalies, Genetics, Early life health

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 make precisely engineered mouse models of genetic variants associated with human congenital anomalies.

To identify how these genetic variations contribute to functional changes during life, across multiple organ systems, and how these changes contribute to human disease.

To improve automated live monitoring of early life in our animal models, which will help us to better understand the consequences of these genetic mutations during the critical postnatal period.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could

be short-term benefits within 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 1 in 20 babies are born with severe anatomical malformations. Each year this equates to 8 million affected newborns, of which 300,000 die within the first four weeks of life. With recent advances in sequencing technology, we are accelerating the identification of possible disease-causing changes in the genetic code of these patients. However, it is a major challenge to prove which of these genetic changes, also called variants, do cause these malformations, as well as to establish the cellular mechanisms by which these changes disrupt normal development.

Novel mouse models will also allow us to monitor disease progression throughout life and in the long term may serve as platforms for developing much needed therapeutic interventions. Our programme will improve the use and data collected from animal models, while advancing the basic research into early life anomalies. Cell-based experiments are often unable to mimic human organ function. By reproducing human gene variants in animals, we can directly examine the effects of human patient mutations into living models, which will lead to more precise understanding of human disease. We will be able to improve our discussions on genetic cause and effect together with clinical geneticists, medical teams and patient groups. The ultimate hope is to provide improved diagnoses and prognoses for patients with congenital anomalies.

What outputs do you think you will see at the end of this project?

Results from this study will advance our knowledge of genetic changes leading to common and rare congenital anatomical anomalies. These may include anomalies such as craniosynostosis (premature fusion of skull bones), neural tube or spinal cord defects, cleft palate and other common disorders such as those that affect heart and kidney function.

We aim to improve the efficiency of linking genetic changes to disease manifestations, which will improve diagnoses, and with time, develop methods of prevention or therapy. This work will allow us to identify new genetic factors and to develop therapeutic platforms for testing new treatments and therapies.

Our major outputs will include new information for the public, patients, clinicians and researchers. This will take the form of peer-reviewed publications and presentations. We will also generate new mouse lines that will be useful to researchers interested in specific gene functions and to clinicians, researchers and industrial partners who can develop therapeutic strategies.

Outputs from this work will include:

- standardised data collection describing disease-related phenotypes, which will be user-friendly and freely accessible online.
- reliable and replicable results incorporating best practices from national and international experts. publications describing the importance of human gene variants in development.
- ay summaries describing new information on gene variants of unknown significance. presentations at conferences and engagement with international collaborators.

engagement with relevant patient organisations and with industrial colleagues.

Who or what will benefit from these outputs, and how?

Information on gene variants will:

- in the short term, improve the clinical diagnostics of rare diseases improve understanding of genetic causality of phenotypes improve the clinical interpretation of patient conditions
- improve prognoses by improving our understanding of disease progression
- in the long term, improve the well-being of patients with congenital anomalies, and for their families, clinicians.
- identify new avenues for potential therapeutics

Publications describing the biological processes in embryonic, foetal and postnatal development

- will add to our (currently limited) knowledge of the fundamentals of human and animal development
- will inform the bioscience and clinical communities of key findings, including negative data, relevant to human development
- will lead to new innovations in diagnosis, prognosis and therapeutics

How will you look to maximise the outputs of this work?

We will aim to maximise the outputs of this work by: publication of findings in open-access journals collaboration with international teams through joint grants
knowledge exchanges by presenting at specialist and public fora

- work with patient organisations and the general public to share our new knowledge incorporate the patient experience in our knowledge gathering
- host visiting students and scientists within our team contribute our findings to public databases
- sharing resources (e.g., protocols, data, animals, tissues) with other researchers

Species and numbers of animals expected to be used

- Mice: 26000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 an incredibly powerful system for understanding mammalian biology. There are now many technologies available for genetically modifying mice, which allows to track specific cells and tissues and to perform experiments aimed at understanding the role of genes in human development. Many of our experiments involve breeding mice with the appropriate genetic changes and taking tissues under terminal anaesthesia. Thus, the animals do not suffer. In some cases, we will need to experiment on juvenile or adult animals in order to understand how the genetic changes affect biological functions such as breathing and eating.

The advanced knowledge and technologies to make genetically modified mice efficiently allow us to reach project aims quickly and precisely (as opposed to rats). Resemblance between human and mouse genomes allow the development of robust mouse models with very similar genotypes and phenotypes to humans (translate well to humans), while the short life cycle of mice allow efficient prediction of future phenotypes.

Typically, what will be done to an animal used in your project?

For the majority of the project, we are using these mice for breeding purposes, which are natural and therefore should cause no adverse effects. Because of this, many of our protocols are of mild severity.

In some rare experiments we administer compounds that alter development or allow us to see cells in a microscope. Such agents may be given in food or water, by gavage or by injection. Administration of compounds is also routine and should cause no adverse effects. Mice will experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal).

We plan to make genetic alterations that will model human disease genes. The generation of new genetically modified animal lines involves killing of some animals and surgical procedures (for example, surgical embryo transfer). In this case, we may perform surgery on recipient females so that genetically engineered embryos can be implanted and grown to full term. We may also induce pseudopregnancy in females.

Most offspring will need to be genotyped, this is typically performed by taking tissue samples, such as ear notching.

Animals will be monitored for discomfort and will be killed by a Schedule 1 method if discomfort becomes evident. Humane endpoints will be determined for each protocol.

What are the expected impacts and/or adverse effects for the animals during your project?

While some of the genetic modifications will have no obvious effect, others may produce phenotypes in the animals. All genetically engineered animals will be monitored closely to mitigate any unanticipated welfare concerns. Newly engineered lines will be monitored particularly closely as it is sometimes difficult to anticipate the effects of specific genetic modifications on individual animals. We expect that many of the developmental abnormalities will be seen in the embryo, and lead to death in utero.

We expect a small percentage of those born will have a likelihood of adverse postnatal effects, which we will predict based on our knowledge of the patient phenotypes. For each line, pilot studies will be used to determine specific endpoints.

For example, newly engineered lines may result in newborns with a severe phenotype such as cleft palate (who are therefore unable to feed). In those cases, we will perform the majority of our analysis during embryonic and foetal stages so that animals will not suffer adverse effects postnatally.

Some animals will exhibit mild adverse effects such as excessive tooth growth, these will be monitored and effects will be ameliorated, e.g. by clipping of teeth.

In some rare cases, animals will be born with developmental abnormalities affecting function. These animals will be monitored in pilot studies to determine humane endpoints including assessment of weight loss, inability to ambulate, laboured respiration, 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)?

We expect that the majority of our animals will be used for breeding and generation of embryos. We expect minimal impacts, with 10% mild and 90% sub-threshold.

We anticipate that the establishment of new genetically modified lines will lead to some unanticipated phenotypes, which will need to be carefully monitored. We expect of these animals, approximately 25% may be of moderate severity, 25% mild and 50% sub-threshold.

Once lines are established and welfare concerns are documented, we expect that we can reduce the severity levels by defining suitable experimental endpoints.

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal models provide a great deal of information on the causes and progression of congenital anomalies. In particular, animals carrying genetic variants are necessary for studying the molecular events underlying human development.

These kinds of studies need to be performed in vertebrates where analogous genes are implicated in the development, healing and regeneration of analogous structures. For example, the distinct bones that comprise the human skull vault are equivalent to the bones in the skull of the mouse, not just in function but also in embryonic origin and molecular identity. Furthermore, the three-dimensional development of the human skull is greatly influenced by surrounding tissues, such as the underlying brain and meninges. While we can test interactions in a limited way in a tissue culture dish, or by computer simulations, we still need to return to animal models to truly test our hypotheses and to understand the impact of genetic mutation on biological function.

Which non-animal alternatives did you consider for use in this project?

In some cases we can replace animal experiments with alternative methods such as human stem cells, which can be created from patient tissues, or can be genetically engineered to carry human disease genes. We sometimes use other non-regulated models, such as yeast or fertilised chicken or frog eggs, to model gene function. We are also working to improve computational models of development, which can help us reduce and refine our experiments as well as narrowing down hypotheses.

Why were they not suitable?

Computational models are also very exciting, but these models need to be rooted in real, functional biological systems. Similarly, in vitro culture of human stem cells are very promising, and in the long- term we are developing approaches to use patient-derived stem cells to study these processes.

However, technologically, it is currently still impossible to model something like an intact functioning animal using human stem cells, as we cannot create a three-dimensional system mimicking form and function of organs or complex structures such as the head. In order to use the human stem cells in the future, we need to better understand how these structures develop in live animals; hence our need for mouse and other models. For early embryonic stages of development, we can use non-regulated approaches such as fertilised chicken and frog eggs, which allow examination of functioning embryonic tissues. However, to truly model the postnatal consequences of human genetic variants, mouse is the most genetically tractable mammalian 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?

Pilot studies will be performed with control wild-type animals, with minimum numbers estimated with the help of a statistician.

For the majority of our experiments, we will be able to use littermate (sibling) controls to minimise variability between animals. Samples sizes for our experiments will be estimated from past experiments, with minimal numbers of animals used whilst ensuring that results are statistically significant.

Total numbers of animals needed for breeding have been estimated based on prior experience with these protocols.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We employ 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.
Where appropriate, 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 will share tissues with our project partners in order to maximise research application and minimise unnecessary use of animals. Sperm and eggs generated in this project will also be stored for sharing with other researchers, in order to minimise generation of new animal lines.

At the end of the experiment, we will harvest as many tissues as possible at post-mortem. If we don’t need to analyse the tissues immediately, we will freeze them and make them available to other researchers working on similar questions.

Following the best guidelines and protocols and publishing robust and reproducible results will ensure that experiments do not need to be repeated.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In order to model human congenital anomalies, we need a genetically tractable mammalian system that can mimic the human life cycle. In this project we use mouse,

which as a mammal is our model that is most neurophysiologically similar to humans. The mouse remains the most powerful system for studying mammalian genetics. One of our aims is to improve early life monitoring of juvenile mice, which will improve our data collection and refine our uses of mice.

Why can’t you use animals that are less sentient?

For our experiments, we first test our protocols and reagents in vitro, to ensure refinement of our approaches. This also reduces the number of animal experiments by minimising our need for troubleshooting in vivo.

As further refinement, many of our experiments are conducted on animals at an immature life stage (e.g. embryonic rodents, fertilised frog or chicken eggs). These immature stages are easily genetically modified and easily studied. When we need to mature these animals, we monitor them carefully to the appropriate stages. The majority of juvenile and adult animals used in this study are for use in breeding colonies, which cause minimal harm. We also breed together strains of mice that carry different mutations, allowing us to study genetic interactions in biological processes. Mating and caring for these animals is generally routine.

While we do not anticipate this, in some cases, genetic modifications may cause phenotypes of moderate severity. We do have to use live animals, because it is important to study how tissues within an intact living organism.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

One of our goals in this project is to improve video monitoring of early life. This will greatly refine our use of mouse models and also improves the relevance to our study of neonatal conditions, which have been difficult to monitor in mice. In addition, we are skilled in using genetically modified animals to generate tissues and cells for studying cellular and molecular pathways in vitro. This allows us to maximise use of our animal models. Finally, we plan to broaden access to genetically modified mouse models of human disease by working closely with collaborators allowing distribution and analysis of animals both nationally and internationally, which will reduce numbers and refine our experiments.

In some cases, genetic modification will produce mouse lines that have deleterious effects (for example, cleft palate). In those cases, rather than allowing the animals to be born, we will cull them in utero so that we can use their tissues for study rather than allowing them to suffer.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We regularly review and update current best practice. We will follow published guidelines such as the PREPARE guidelines.

We consult current published references include:

Animals in Science Regulation Unit (ASRU) Guidance and regulatory advice, such as:

Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes

Guidance on Breeding Protocols for Genetically Modified Mice Guidance from LASA www.lasa.co.uk

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, we will meet the NC3Rs Regional Programme Manager, and attend Regional 3Rs symposia.

We participate in national and international meetings where group share best practice on genetic engineering approaches. This keeps us informed on current advances in animal welfare and research and also allow us to efficiently share any improvements that we make in our project.

58. Development of novel therapeutics for pain

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Pain relief, Inflammatory pain, Neuropathic pain

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 purpose of this project licence is to increase our knowledge and understanding of the mechanisms involved in the pain pathway and to use that knowledge to identify and test new candidate molecules in relevant models. This will ultimately help us develop new potential treatments for 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?

Pain is defined as ‘an unpleasant sensory and emotional experience associated with or resembling that associated with actual or potential tissue damage’ and is represented by various conditions such as low back pain, neuropathies (where the pain is due to damage

to nerves), and arthritis (joint pain). Chronic pain has been reported to be one of the most prominent causes of disability worldwide.

The treatment of both inflammatory pain (in which the pain is caused by tissue damage) and neuropathic pain is currently poor and is considered a major area of unmet need. Despite drugs being available for the treatment of these conditions, less than half of all patients achieve adequate pain relief from current medications. Existing therapies also have major side effects (sedation, nausea, vomiting) which can limit their effectiveness, and discourages patient compliance. Physicians generally agree that even small improvements in safety and/or tolerability would be good progress. Any novel therapies would be expected to have better profiles than existing treatments.

Our team has been focused on developing novel treatment options for a variety of painful conditions with a few compounds developed under previous similar licences being trialled in the clinic currently. We perform a large amount of in vitro (using cells in the laboratory) experiments to understand the mechanisms of pain, develop efficient compounds and test the efficacy of these compounds in relevant disease animal models to give us an extra layer of confidence in developing these new medications.

Through continuing this work, we intend to develop analgesics (pain medicines) which are more efficacious and safer than the current medications.

What outputs do you think you will see at the end of this project?

New candidate drugs/compounds (those that are being developed and trialled but have not been approved for use yet) for inflammatory and neuropathic pain and an increased understanding of the mechanisms responsible for these painful conditions will be the main outcomes of this project licence.

By testing the effects of novel compounds on either the development or treatment of pain, we hope to identify and develop new therapies for painful conditions such as arthritis and neuropathies. Tissue collection during the studies will increase our understanding of the mechanisms involved in painful conditions and validate our targets. This may also help with the development of biomarkers, which are currently lacking for a majority of analgesics used clinically.

Additionally, findings will be disseminated in the form of papers or presentations at conferences to help advance the field. Some of the work may also contribute towards patents for new compounds. Any unsuccessful approaches may be published in open access platforms, as long as it does not contain confidential data.

Who or what will benefit from these outputs, and how?

The work being undertaken under the realms of this licence will be of benefit to the wider scientific and medical community, and eventually patients. The primary potential benefit from work carried out under this licence is the development of new and better treatments for painful conditions. Any new treatments which come from this work would be expected to show better efficacy and tolerability profiles (resulting in lesser side effects) than existing treatments.

The short-term benefit of the work undertaken will be progression of pain projects through the various stages of drug discovery, starting from target validation to the development of a candidate drug to conduct clinical trials.

Most benefits arising from the work being undertaken will be observed in the long term (10-15 years) due to the time scale required to develop new medicines. For example, compounds that were tested under the forerunners of this license in the past decade are now in Phase 1-2 clinical trials for chronic pain.

It is well known that multiple diverse mechanisms are involved in pain pathways leading to a complex pathology, which is still largely less understood. Except for the gabapentinoids class (analgesic to treat neuropathic pain), there has been little development in new pain medicines over the past decade.

Recently, the development of targeted therapies such as inhibitors of nerve growth factor (NGF, which is an important mediator of nerve growth), have shown remarkable efficacy in clinical trials. Despite being challenged by safety issues this approach has helped set in motion a revolutionary change in the treatment of chronic pain.

How will you look to maximise the outputs of this work?

Our team works in collaboration with many different academic and pharmaceutical institutes to help with the progression of the field. Data will be presented where appropriate and where internal confidentiality permits, at both national and international scientific meetings and conferences. We also expect to publish results, not subject to confidentiality, in peer-reviewed journals for the benefit of the wider scientific community. Where possible, we will publish results on platforms that have open access.

Species and numbers of animals expected to be used

- Mice: 7000
- Rats: 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.

While most of the studies carried out under this licence will be using mice, a few studies must be undertaken using rats. Testing in rats will allow us to provide information, for example, efficacious doses which can be used in future studies such as those assessing the safety of the drug, which are required for regulatory approval following the selection of candidate drugs. There is wide literature supporting the use of mice and rats in models of pain, which will be used to provide a background to the studies prior to instigating any work. We may also use genetically modified mice in all procedures, which will enable us to better understand pain pathways and provide information about the selectivity of test substances. All studies will be performed using adult rodents as the models we are using are representative of clinical conditions such as arthritis and neuropathic pain that

commonly occur in adults. These models have been well established for decades, while also undergoing refinement when new information about human disease pathology or animal models is available.

Typically, what will be done to an animal used in your project?

Any animal arriving in our animal unit will be allowed to acclimate in their home cages for 7 days, with food and water provided ad libitum and general daily checks being performed. Most of our studies will involve animals being microchipped for identification and blinding purposes, following which they will be allowed to recover before carrying out any procedure.

Inflammatory pain models: The typical animal will undergo dosing with substances causing inflammation into the foot, ankle or knee joint, following which the animals will be evaluated for the desired behavioural endpoints at various times post-dose. Behavioural measurements may range in time from a period of a few minutes in the acute models, through to 4-5 weeks in the case of the more chronic joint pain models. Once a pain-like behaviour is induced, the animal will be dosed with compounds that are being developed to treat pain, to investigate if they can reverse the pain behaviour in the animal. If a compound is being developed to prevent pain, it may be dosed before or alongside the inflammatory substance. During or at the end of the experiment it is anticipated that samples (such as tissues or blood) may be taken for subsequent analysis such as biomarkers (a substance found in blood or other tissues that can indicate the presence of a disease) and pharmacokinetics (how the body interacts with the administration of the compound). In the acute models of inflammatory pain where interventions may be performed as early as 30 minutes after foot injection of the inflammatory substance, using an analgesic will interfere with the scientific outcomes and hence will not be employed. However, in the chronic models involving the ankle or knee joint, no behavioural testing will be performed in the first 3 days after dosing of the inflammatory substance. We will hence employ local analgesia to help with recovery if no interventions such as tissue collection is required in the immediate 48 hours after dosing of the inflammatory agent. However, we will consider running pilot studies every time a new target is being investigated to determine the effect of local analgesia on tissue collectionand analysis to determine any biological effects. If none are observed, we may consider using local analgesia.

Neuropathic pain models: The typical animal will undergo nerve ligation surgery (where the sciatic nerve will be ligated using a suture) under general anaesthesia or the administration of a chemotherapeutic agent (anti-cancer drugs, administered into the blood vein or abdomen commonly), following which the animal will be evaluated for the desired behavioural endpoints at various times post-surgery or dosing, which may last until 4-6 weeks. In the surgical model, the animals will be allowed to recover for 3 days before any behavioural testing is performed. As the chemotherapy model will involve multiple injections of the chemotherapy and this is not expected to cause any distress to the animal, they may be tested at any point in the study. Once a pain-like behaviour is induced, the animal will be dosed with compounds that are being developed to treat pain, to investigate if they can reverse the pain behaviour in the animal. If a compound is being developed to prevent pain, it may be dosed before or along with the inflammatory substance. During or at the end of the experiment it is anticipated that samples may be taken for subsequent analysis such as biomarkers and pharmacokinetics. In the surgical nerve ligation model, we will employ peri-operative analgesia to ensure smooth recovery

from the surgical procedure. The animal will be allowed to recover for 3 days before any behavioural testing is performed. However, if any interventions such as tissue collection is planned within the 48 hours after surgery, no analgesics will be utilised as it may interfere with the study outcomes. However, we will consider running pilot studies every time a new target is being investigated to determine the effect of peri-operative or local analgesia on tissue collection and analysis to determine any biological effects. If none are observed, we may consider using them. If in any instance, an unexpected drug interaction with any novel compound is observed, the peri-operative analgesia regime will be reviewed to identify alternatives to avoid this in further studies. No analgesia will be employed in the chemotherapy induced peripheral neuropathy model, as such measures are not available in the clinic and the animal may be tested any time after administration of the chemotherapy.

All behavioural tests employed are used to either measure a painful response to a mechanical or heat/cold stimulus. Specified cut off points are used to avoid adverse effects of testing. These tests are animal equivalents of the measures that are used to assess pain in humans. All models mentioned have been refined to cause the least suffering. From our experience, the animals are generally active without any signs of distress, apart from those expected due to the nature of the model.

What are the expected impacts and/or adverse effects for the animals during your project?

Inflammatory pain models: Due to the nature and administration of compounds, the common adverse effects that may occur are redness, swelling and mild discomfort at the site of administration. The injection itself and administration of control substances are not expected to cause any harm, apart from mild and transient discomfort. The animals are expected to develop a pain-like behaviour in the form of mechanical and thermal hypersensitivities (reduced threshold to feel pain, pressure, heat and cold).
However, from previous experience, this does not affect the normal behaviour of animals.

Neuropathic pain models: From previous experience, animals are expected to show an uneventful recovery from the nerve ligation surgery, similar to that seen in humans undergoing surgery under general anaesthesia. Problems with wound breakdown caused by animals licking the site of surgery and lameness in the affected limbs are most commonly seen over the first few days. Following this,
except for mild gait changes, very few adverse effects are ever seen such as wound opening. However, these do not have any effect on the overall welfare of the animal. The animals are expected to develop a pain phenotype in the form of mechanical and thermal hypersensitivities. In the case of administration of a chemotherapeutic agent, the animals may be expected to show signs of transient discomfort and pain-like behaviour such as mechanical and thermal hypersensitivities. Additionally, some swelling and redness at the site of chemotherapy administration may last for a few days. No effect on the normal behaviour of the animals is expected.

Unless otherwise specified, the administration of substances and withdrawal of body fluids will be undertaken using a combination of volumes, routes and frequencies resulting in no more than transient discomfort and no lasting harm. These tests are not expected to produce any adverse events. The behavioural endpoints used to measure mechanical and thermal hypersensitivities are not expected to have adverse effects. To further refine the

process, we have specified cut off points to prevent any injury or tissue damage due to testing.

The adverse effects mentioned above are all expected to be transient and resolve over a few days without affecting the well being of the animal. If any adverse effects beyond that expected occurs, the NVS will be consulted depending on the nature of the adverse effect. Any severe adverse effects resulting in an alteration of appearance, 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 – 10%
Moderate – 90% Rats:
Mild – 10%
Moderate – 90%

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 animal models of acute and chronic pain conditions is fundamental to providing insights into molecular, cellular, and systems organisation of pain that can otherwise be hard to determine clinically. These animal models have been designed to capture either some or most of the characteristics we see in the clinic with patients suffering from various painful conditions. Such animal models offer the ability to determine the analgesic efficacy of novel compounds.

Non-animal alternatives such as in-vitro testing of neurons have been extensively studied by many groups and show that a proportion of the fundamental properties are lost in culture, which leads 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. Often, retaining one of the fundamental properties is insufficient to inform us about the prevention of pain due to the complexity of the systems involved in the pain pathway. In addition, testing in culture systems alone cannot provide information about the efficacy of drugs or the severity of pain, 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 to study arthritis have been gaining traction. Although they do a particularly good job of being able to study interactions between different

cells/tissues, they are unable to fully recreate the entire joint environment, which may lead to false results, and ultimately the development of either an unsafe or ineffective compound for human use.

As a result, we carefully perform and design these animal experiments to obtain valuable information about translation in the clinic, which cannot be achieved by non-animal alternatives. Where possible, extensive in-vitro experiments are performed to understand more about the target and characterise responses produced by compounds, to help choose the best ones to be evaluated in these animal models.

Which non-animal alternatives did you consider for use in this project?

In-vitro systems have been and will be used by us as replacements wherever possible to examine selected aspects of the targets that we aim deeply investigate. Preliminary studies will be conducted in a range of in-vitro cell assays, using cells relevant to the disease we are targeting. Any substance which is selected for testing in animals will have been examined in a number of these in-vitro tests to ensure that it has the required selectivity at the target site and has the desired affinity (the degree to which a compound may interact with the target) for the target. Where available, comparisons will be made with data obtained from other drugs in the same class to determine which better satisfies the criteria for development. Additionally, within the team, we have access to over a decade's worth of data regarding all the compounds that have been developed for various conditions. If a target has a large body of evidence in the literature, an extensive review will be performed to gain a thorough understanding of the current scientific field.

Why were they not suitable?

Pain is a highly complex process requiring input from many parts of the nervous system and so, for this reason, in-vitro testing alone is not enough to determine if new compounds will be effective analgesics. Furthermore, the use of animals will help determine if novel compounds produce an analgesic effect, which is impossible to deduce from in-vitro systems. No currently available in-vitro systems can fully replicate human physiology, as stated above. We have fully acknowledged their strengths, reviewed their use, are aware of and appreciate their limitations as these studies may be valuable, but cannot completely replace what behavioural studies can tell us. The behavioural tests that we use have been carefully refined to fit each model and will be chosen based on the scientific outcome expected. Most of these tests mirror the tests used clinically to assess pain in humans. The amount of information we can obtain from carefully designed in-vivo 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 where available and use modelling to project occurrences using available datasets.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 throughout the project has been based on numbers used in previous licences of similar nature. We have a statistician available within the organisation who will help us calculate sample sizes using historic data. 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?

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.
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 are used and that the correct analysis is carried out to give the best possible interpretation of the results.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where newer study designs and outcomes are being considered, pilot studies will be run to obtain preliminary data, based on which the design of future studies can be determined. Drug treatments are randomised based on pre-dose readings by dividing animals into relevant groups with approximately equal scores. Measurement of treatment effects is carried out with the operator blind to treatments to minimise bias. In all cases, studies will be carried out to the principles outlined in the ARRIVE guidelines.

In the previous licence, we have trialled the use of tail vein microsampling to extract blood for biomarker analysis, which has been successful and subsequently resulted in a significant reduction in the number of animals required for such studies.

Following discussions with the local Animal Welfare and Ethical Review Body (AWERB) and with other organisations, we have realised that there is a drive to reduce the use of surgically prepared sham animals. For this reason, and to reduce animal usage further, we have decided to no longer perform sham surgery in our nerve ligation model. Control groups must be used in compound studies due to the nature of vehicles being variable between each drug developed. Where possible, the control groups can be avoided if a previous study has shown no effects. In addition, we will utilise tissues and samples from our studies to benefit other pain-related projects so we can maximise our scientific output.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The models we have chosen to use in this licence include those which we believe are minimally invasive to the animal yet will provide us with the most information. We have consulted with clinicians within the organisation to pick the most appropriate models where possible and intend to use those which model important clinical features of the disease states. These models have been extensively studied in the literature and have been validated using compounds which are in clinical use for the treatment of inflammatory and neuropathic pain.

Only one sex of animals (usually female) have been used in most of our previous studies enabling us to control, as far as possible, between experiment variability. In addition, female mice can be group housed throughout the length of the studies whilst males would require rehousing for welfare reasons due to fighting etc., which is not beneficial for our studies. This fighting can induce stress which in itself can affect the pain response. This will also avoid excess animal wastage in the breeding of such animals. Additionally, having both sexes in studies involving behavioural readouts adds an additional complexity in conducting these studies due to interference with the same operator having to handle them and test on the same instrument. In our previous experience of using both sexes, we have had misleading results with behavioural testing. Benefits from the studies are accrued to the wider population despite only conducting tests in one sex of animals. Discussions with clinical scientists within our group reveal that testing in single-sex groups is not seen as a problem at the proof of concept and efficacy evaluation stage, as covered by the work in this licence. Once a compound enters clinical testing both male and female patients are used and are balanced across treatment and placebo groups. To date, no compound developed from work carried out under the forerunners to this licence has shown sex differences in its effect in clinical trials. However, we will continue to conduct pilot studies to trial different methods of including both sexes in behavioural studies for the entire period of this licence.

Throughout the study period, all animals will undergo daily health checks and be weighed weekly to ensure their welfare. Additional health checks and weighing will be carried out where necessary, for example, after procedures. Due to the chronic nature of the conditions in the clinic, it is necessary to follow the animals for a few weeks to replicate the human disease processes. However, care will be taken to ensure the severity limits set out for each of the models are not exceeded.

Why can’t you use animals that are less sentient?

Since most of the pain indications that our group focuses on are commonly seen clinically in adults, all our studies would prefer to use adult animals. A majority of our studies require identifying pain-like behaviour which can be measured using well-validated endpoints that

cannot be assessed in less sentient or terminally anaesthetised animals due to their reduced capacity to perceive pain.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All the models in this licence are designed to mimic some, if not most, of the symptoms observed in human conditions and hence some level of pain is inevitable. However, experience gained from previous licences has shown that the level of pain (indicated by the assessed pain-like behaviours) experienced by the animals is not such as to cause any major changes in the welfare of 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 treatments. Often these animals can also be seen climbing grids and running around in their home cage, as early as a few minutes after surgery or dosing. Observations have revealed that the level of activity and general movement seen in treated animals is also no different from that observed in untreated animals. The insults used in the models have been assessed to be the minimum which can be used to cause a significant reproducible biological effect without causing excess adverse events. Measures to reduce animal stress will be undertaken throughout the study period. All animals will be allowed to acclimatise to the study environment and operator before performing any procedures, especially behavioural tests. They will be provided with sufficient enrichment in the cages, which from our experience also helps with recover post-surgery and reducing the incidence of wound licking which may lead to open wounds and infections. In most cases, animals will be socially housed in groups. All animals will be checked daily and weighed weekly, irrespective of the nature of the insult. Based on the model being run, additional monitoring, weighing and health checks will be considered to ensure adequate recovery from any dosing, procedure, or surgery and adherence to humane endpoints. When performing studies involving surgery, we will provide the animals with recovery gel and soft bedding as part of post-operative care. In all new studies undertaken, the level of insult will be assessed before conducting further studies.

Since most studies will involve behavioural endpoints, the animals will be habituated to the instrument and the experimenter before the start of the study.

Previously we have not employed peri- or post-operative analgesia as we believe that the use of analgesics during the development of neuropathic pain following surgery interferes with the mechanisms required to induce the pain symptoms we are investigating. However, we are aware of a small but increasing literature regarding the use of postoperative analgesia in neuropathic models suggesting that no subsequent effects are seen. These studies concentrate on the development of neuropathic hypersensitivity and little or no mention is made of subsequent effects following pharmacological intervention such as the studies covered under this licence. We have therefore conducted pilot studies to investigate the usage of peri-operative analgesia in the surgical neuropathic pain model and observed no difference in the pain phenotype developed of the efficacy of gold standard analgesics used. We will hence employ peri-operative analgesia in the surgical nerve ligation model and local anaesthesia in the inflammatory joint pain models to aid smooth recover from surgery and dosing, respectively. The exception to this would be if any interventions such as tissue collection is planned in the first 48 hours after surgery or dosing of the inflammatory compound, as it may interfere with the scientific outcomes as shown in literature. However, we will consider running pilot studies every time a new target is being investigated to determine the effect of local analgesia on tissue collection and

analysis to determine any biological effects. If none are observed, we may consider using local analgesia.

Although we do not expect any major drug interactions, if any novel compound we develop results in alterations of the scientific outcomes due to interaction with peri-operative analgesia, the analgesia regime will be revisited to look for alternatives to avoid drug interactions, while also ensuring adequate post-operative recovery for the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the resources available such as the guidance and publications from the NC3Rs, PREPARE and LASA to inform us of any refinements that can be utilised in our studies. Where possible, we will also consult resources such as SYstematic Review Center for Laboratory animal Experimentation to improve the rigour and translatability of 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 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 Institute of Animal Technology (IAT), and the Royal Society for the Prevention of Cruelty to Animals (RSPCA) will provide additional sources of new recommendations.

59. Identifying and modulating new targets for improved cancer treatment

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Therapy, Ionising radiation, normal issue, Immune 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?

To identify new ways of detecting cancer earlier and to find better and safer treatments.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Approximately 1000 new cancers diagnoses, and 460 cancer-related deaths occur in the UK every day. This disease is therefore a significant public health problem. In order to improve cancer treatments and increase survival rates for those diagnosed with cancer we need to find ways to detect cancer earlier and also be able to treat the disease with better treatments. As the number of cancer survivors increases due to better treatments it will

also be important to ensure the quality of life of those survivors is not adversely affected by side effects of treatments. Improving the safety of current and future cancer treatments is therefore also very important. With this protocol we are seeking to identify and test new tools or markers that can one day help doctors detect cancer earlier. We also seek to find new targets that can improve current standard-of-care treatments (such as radiotherapy). This includes reducing side effects experienced by children undergoing these treatments**.** Our interest is to identify proteins that we can target with drugs to make radiation work better at killing the tumour but at the same time reduce the side effects of irradiation in surrounding healthy tissue.

What outputs do you think you will see at the end of this project?

New information, with particular reference to better treatments for cancer and potential early detection tools. This will include new therapeutic targets and/or potential drugs that could be used in future clinical testing to improve the effects of radiation on tumour response without increased toxicity.

New information will be disseminated in publications and also in public engagement events aimed at dissemination of outputs to members of the public (including local members of our community).

Who or what will benefit from these outputs, and how?

In the short- to medium-term, colleagues in the scientific community will benefit from the new information and publications produced. The pharmaceutical industry will likely also benefit from the new therapeutic targets suggested from our work. Members of the public may also benefit from our dissemination of the work in public engagement and outreach activities.

In the long-term we hope that some of the therapeutic targets or early detection tools that we identify may be tested to evaluate their potential in the clinical setting. This would benefit patients. However, for most of our work the time frame for this benefit is likely to be outside the scope of this project.

How will you look to maximise the outputs of this work?

We will continue to collaborate with others (locally, nationally and internationally) to ensure that dissemination of our work/knowledge is as widespread as possible and happens as quickly as possible. For example, collaborations often can speed the time to publication of findings when two or more groups join efforts to achieve a common goal. We will also ensure that resources are made available to other researchers (e.g., data, animals, tissue). We will always seek to publish our findings in open-access journals, and we will upload our manuscripts to BioRxiv to make them available prior to publication (to accelerate access by the scientific community). Our work will also be disseminated by social media platforms (Twitter, LinkedIN).

Species and numbers of animals expected to be used

- Mice: 7100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 identify new or improved anti-cancer therapies, we need to reproduce tumours in mice to mimic those occurring in cancer patients, and treat animals with clinical approaches, which may cause adverse effects. We need to use model organisms such as mice since it would be unethical to perform these experiments in humans. Therefore, experiments need to be performed in living organisms to account for toxicity to different organs and to account for the effect of the immune system on these responses. It is difficult to accurately reproduce the full effects of the immune system on tumour responses with non-animal alternatives since the different cells and molecules of the immune system work together in ways which we still don't fully understand so it is difficult for us to reproduce these conditions accurately in vitro.

Mice are used as they are genetically and physiologically similar to humans. Mice are the most widely used organism in cancer research and there are a wide range of genetic and pharmacological tools available for studies in mice which accelerates research and reduces costs.

We may also use genetically altered mice, specifically strains of mice lacking particular components of the immune system. These mice will help us identify the impact of these different parts of the immune system on tumour response or normal tissue toxicity to treatment. The majority of these genetically altered strains have either no or a mild phenotype and would therefore not be expected to cause harm to the animals.

Most of our work will focus on adult cancers so we will use mice of approximately 6-8 weeks of age for our studies. Both males and female mice will be used unless this is not appropriate (for example when studying early detection tools for ovarian cancer where only females will be used).

Some of our work on identifying safer and better treatments can be particularly relevant for paediatric patients so we will occasionally also use infant and/or juvenile mice for these studies.

Most of our work will involve the study of gastrointestinal, brain or ovarian tumours where there is currently a need to identify safer and more effective treatments as well as methods for detecting cancer earlier. However, other tumour types (where safer and more effective treatments are necessary) could also be investigated in the future.

Typically, what will be done to an animal used in your project?

Typically, animals will experience mild, transient pain and no lasting harm from administration of substances or cells by injection using standard routes (e.g. oral gavage, intravenous, subcutaneous, intraperitoneal). Animals may also experience mild transient pain from injection of tumour cells under the skin or intraperitoneally under anaesthesia. We will also inject tumour cells into the brain following surgery under anaesthesia which

may lead to moderate transient pain. This is mitigated by administering analgesia prior to induction. Additional analgesia will be given if signs of pain become apparent.

Genetically altered mice will be bred that lack genes that will affect the immune system. These phenotypes could make the animals susceptible to infection, but we will mitigate this by housing them in bio secure caging systems. Of note, we have previously bred mice lacking the immune receptor C5aR1 and these mice have no obvious detrimental phenotype. We anticipate that the majority of the breeding undertaken in this protocol will involve mice lacking immune components such as C5aR1.

Genetically altered mice that are susceptible to tumour growth may also be bred. For some of these strains the mice may develop pale feet, ears or mucous membranes. Such mice are therefore flagged for close observation, and should they develop further signs and symptoms of tumours such as hunching, weight loss or lethargy, are killed by schedule one methods.

Mice may be exposed to an irradiation source to evaluate the effects of irradiation on tumour response and healthy tissue toxicity. This is normally done under general anaesthesia as we need the animal to remain still whilst we target specific tissues.

If the irradiation is aimed at the brain, a change is expected in the brain mass along with some reduced movement or potential signs of tiredness. We will assess changes to behaviour or tiredness following treatments such as irradiation using behavioural, running wheel or treadmill tests. Running wheel tests will require the mice to be single housed. In the majority of instances these tests will involve spontaneous assessment of behaviour (e.g. measures of spontaneous locomotion, exploration, social interaction, tests of habituation such as spatial novelty preference and object recognition, consumption of palatable food or liquid). Occasionally, we will need to motivate the mice to perform certain tasks by briefly restricting their water consumption (for a maximum of 28 days before a rest day is provided).

These tests may need to be repeated to assess the effect of treatment at later timepoints. Rounds of tests will only be repeated once and there will be a minimum of 28 days between rounds. A footshock or loud noise (aversive test) is required for some fear/anxiety based tests. If animals undergo an aversive behavioural test they will not undergo a treadmill exercise test. Aversive or treadmill tests will always be carried out last (after spontaneous or non-aversive tests). In some occasions, animals with tumours might also undergo these behavioural/fatigue tests. Only animals with stable body weights post-tumour induction will undertake behavioural/fatigue tests. Weight and body condition will be closely monitored and animals will be returned to ad libitum drinking to return to their target weight if necessary.

Animals will experience mild and transient discomfort from blood sampling and imaging procedures. Typically any imaging procedures will be non-invasive.

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.

What are the expected impacts and/or adverse effects for the animals during your project?

Superficial tumours may invade the underlying muscle as they grow, which in turn may impede the animal's movement. Animals showing impeded movement that affects their ability to easily reach both food and water will be humanely killed within 24 hours of the detection of tumour invasion. Where tumours invade the skin, a dry scab will form that does not cause the animal any discomfort. Where these tumours progress to form a wet ulcer, we will allow 48 hours for it to dry. If the ulcer does not dry we will humanely kill the animal.

As with human patients, animals that have tumours can experience weight loss of up to 15% compared to their weight prior to the tumour being induced. We would support these animals with moist palatable food to ameliorate the weight loss as soon as their body weight drops to 10% from baseline. Typically, we would expect to see improvement within 24 hours of this intervention. If recovery of body weight is not observed the animal will be humanely killed.

Mice predisposed to intestinal tumours may also be occasionally used. These can present (~50% cases) with pale feet, ears or mucous membranes. This has been the subject of intensive discussion, resulting in very close observation, which has shown that these animals are not distressed and behave normally in the absence of the other signs and symptoms listed above. Such mice are therefore flagged for close observation, and should they develop further signs and symptoms of tumours such as hunching, weight loss or lethargy, are killed by schedule one methods.

For animals undergoing surgical procedures there may be some blood loss, but this volume is not expected to cause any adverse effects. General anaesthesia will be induced using gaseous substances such as isoflurane, which allows for a swift induction and a swift recovery. The duration of any anaesthesia is kept to the shortest duration possible. Tumour induction following surgery (e.g. for brain tumours) may result in moderate pain so animals will receive analgesia prior to induction, and additional analgesia will be given if signs of pain become apparent.

When performing intracranial injections to induce brain tumours there is the potential risk of blood collecting between the skull and the protective lining covering the brain. Animals that experience this will show subdued behaviour and will be humanely killed.

Broad field irradiation of healthy tissue (typically to the brain, bone marrow or abdomen) may result in some toxicity. The toxicity from this exposure will lead to weight loss, diarrhoea, partial hunched posture and potentially some thinning and weeping of skin at the irradiation site. There is also increased risk of infection, but this is mitigated by housing the animals in a bio secure environment.

If the irradiation is aimed at the brain, a change is expected in the brain mass along with some reduced movement or potential signs of tiredness. We will assess changes to behaviour or tiredness following treatments such as irradiation using behavioural, running wheel or treadmill tests. These tests will cause some stress to the animal due to it being handled, and being assessed away from its home cage and normal environment. Introducing a mouse to an unfamiliar environment may cause a stress response such as abnormal behaviour. Mice will be carefully monitored throughout the test and removed from the apparatus if these signs of excessive stress are seen for more than 1 minute. This reaction will be recorded and the mice will not be retested in any other anxiety-

inducing test. Any mice showing a prolonged stress reaction and not returning to normal behaviour in their home cages within 2 hours will be killed. In addition, weight loss may occur following water restriction (required to motivate the animals to undertake the tasks). Animals will be weighed before and during the period of water restriction. Weight and body condition will be closely monitored and animals will be returned to ad libitum drinking to return to their target weight if necessary. In some occasions, animals with tumours might also undergo behavioural/fatigue tests. Only animals with stable body weights post-tumour induction will undertake behavioural/fatigue tests. Any animal that is on water restriction and becomes sick will be immediately returned to ad libitum water.

Water restriction may also result in dehydration indicated by dry, or absence of, faecal pellets, skin tenting, hunched posture, and piloerection.

Imaging techniques to measure and monitor tumour growth will be performed under general anaesthesia and is not expected to have any adverse effects.

During any procedure involving general anaesthesia the animals will be kept warm and hydrated (and eye drops will be used as necessary) with a close monitoring programme incorporated during the recovery phase as you would do with the human patient.

Expected severity categories and the 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 15% Subthreshold, 15% Mild 70% 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?

In order to identify new or improved anti-cancer therapies, we need to reproduce tumours in mice to mimic those occurring in cancer patients, and treat animals with clinical approaches (radio-, immuno-, chemo-, and target therapies), which may cause adverse effects. Therefore, experiments need to be performed in living organisms in order to account for toxicity to different organs and to account for the effect of the immune system on these responses. It is not possible for us to perform these experiments in humans since, besides it being unethical, there is too much variability occurring amongst cancer patients. For these early stages of discovery research, this variability makes it very difficult to easily or consistently investigate the reasons why our new cancer treatments are working the way they are. For us to be able to perform in-depth analysis of our new cancer treatments

we need experimental models that are more homogeneous and that can be consistently exposed to a set of conditions (such as mice housed in controlled conditions).

Mice are used as they are genetically and physiologically similar to humans. Mice are the most widely used organism in cancer research and there are a wide range of genetic and pharmacological tools available for studies in mice which accelerates research and reduces costs.

Which non-animal alternatives did you consider for use in this project?

We will use tumour cell lines and organoids (e.g., tumour and healthy intestinal organoids) as non- animal alternatives. These models are particularly useful for initially testing the effects of drugs or identifying the mechanism of action of certain molecules. Prior to testing in animals, drug doses will be set based on our previous in vitro/organoid studies, or information from Industry/collaborators/literature in order to minimise the risk of toxicity.

Why were they not suitable?

While non-animal alternatives can be useful in initial studies, our work ultimately requires a living organism in order to account for toxicity to different organs. Furthermore, a living organism is necessary to fully study the effect of the immune system on tumour progression and the response of tumours to different treatments.

It is difficult to accurately reproduce the full effects of the immune system on tumour responses with non-animal alternatives since the different cells and molecules of the immune system work together in ways which we still don't fully understand so it is difficult for us to reproduce these conditions accurately in vitro.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

A statistician previously helped us with calculations using typical variations from our own earlier experimentation to calculate minimum numbers of animals to be used whilst ensuring that the results are statistically significant. Sample sizes for our experiments are estimated from past experiments. For experiments where we will assess the effects of a particular treatment on tumour growth, calculations typically show that we need group sizes of 10 to achieve the quality of results we need. For experiments where we are trying to understand how or why mice responded in a particular way, 5 mice per group is typically sufficient to achieve statistical significance.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The experimental design assistant (EDA) from NC3Rs is used and will continue to be used to design experiments. The EDA allows randomisation and blinding of subjects on the same platform. Where possible all data analysis will be performed blind to the assigned treatment groups. Mouse numbers per group will continue to be chosen as stated above and following the 3Rs principles. Reporting of animal experiments will follow the NC3Rs' 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?

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 animals and to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced. Pilot studies will be used to assess variability and calculate minimum numbers of animals to be used whilst ensuring that the results are statistically significant. Each experiment will be designed to maximise the data output generated per mouse used. For example, experiments where tumour radiation response will be assessed over time will also allow assessment of tumour markers by histology at the end of the experiment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

To evaluate the impact of the immune system, mouse strains with an intact immune system will be used. Immunodeficient mouse strains may also be used to assess the impact that absence of a particular arm of the immune system may have on tumour response. Appropriate housing conditions for each strain will be followed to minimise harm and the animals will be housed for the minimal amount of time possible.

For tumour studies we will implant tumours (or use strains susceptible to tumour development) and will monitor tumour growth following best practice guidance to minimise suffering, distress, or lasting harm.

Subcutaneous tumours usually do not invade locally or metastasise to healthy organs, so cause minimal damage to animal welfare.

Tumour induction will be performed under brief anaesthesia to allow better positioning of the tumour. We will also use analgesia prior to induction when administering tumour cells into the brain (following surgery under anaesthesia). Additional analgesia will be given if signs of pain become apparent.

In efficacy studies, tumours will grow up to the minimum size (< 1200 mm^3) necessary to obtain relevant data, in order to minimise clinical signs caused by tumour burden.

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.

To minimise the risk of potential toxicity to animals where tolerable doses need to be established, a small number of animals will be used for establishing tolerated doses.

Local tumour irradiation will be performed by exposing the tumour and surrounding tissue, but minimising tissue exposure outside the radiation field by lead shielding. In addition, irradiation time is short and mice will be anaesthetised during radiation to minimise potential stress and discomfort.

Where we want to assess anxiety or fear in animals following a particular treatment the most refined methods are the use of behavioural tests. These behavioural tests have been refined progressively over decades. We need a battery of behavioural tests to be able to assess the effects of treatment on learning, anxiety, and depression since we do not know exactly what aspects of these phenotypes will be affected by the drug/treatment combinations to be used. We will therefore study spontaneous, appetitively and aversively motivated behaviours. Aversively motivated behavioural tasks are necessary to study anxiety, fear and emotional learning which are central to many human psychiatric conditions such as anxiety and mood disorders. Radiotherapy to the brain has been associated with anxiety and mood disorders and this is why we want to assess the effects of drugs in combination with radiotherapy in our models. For many experiments, we will capitalize on the natural, spontaneous behaviour of the animal for our testing purposes. For example, spontaneous, exploratory-based behavioural tests of unconditioned anxiety reduce the cost to the animal by avoiding the need for appetitive or aversive training stimuli. Although in theory there could be some PSDLH (e.g. in the brightly lit, anxiogenic open field), this will usually be self-limiting as far as the animal is concerned.
Thus, importantly, it is the animal itself that chooses whether to spend time in the more anxiogenic open areas of the apparatus or in the less anxiogenic closed areas (e.g. in the elevated plus maze or light/dark box).

In some instances, we will also use appetitively motivated maze and operant tasks in which animals are placed on temporary controlled access to water (for a maximum of 28 days before a rest day is provided). Restricted access to water is important to ensure the animal is motivated to perform the task.

Rounds of tests will only be repeated once and there will be a minimum of 28 days between rounds. In some occasions, animals with tumours might also undergo behavioural/fatigue tests. Only animals with stable body weights post-tumour induction will undertake behavioural/fatigue tests. Any animal that is on water restriction and becomes sick will be immediately returned to ad libitum food.

Some learning and memory tasks and tests of conditioned anxiety/fear rely on the use of aversive stimuli. For aversively motivated behavioural experiments our collaborators have refined the experimental approaches over many years to minimise potential adverse effects. For example, for aversive learning experiments, shock intensity, duration and

numbers have been optimized to minimize the stress to the animals but at the same time achieve the purpose of the experiment. Shock levels are designed to cause only brief peripheral discomfort (they produce unpleasant prickling sensations in humans) rather than serious pain. The use of the shock is essential for the quantifiable assessment of acquired (conditioned) anxiety and fear and test the ability of the animal to unlearn the association of a tone with a shock. Use of auditory aversion induction alone would therefore not be sufficient to achieve the scientific aims of the test. Importantly, deficits in this behaviour have been linked to areas of the brain that are affected by cancer treatments such as radiotherapy. It is important for us to be able to study these deficits in the context of combinations of radiotherapy and other treatments which may exacerbate or improve these responses. There is no established alternative method available. The shock levels used are carefully calibrated to ensure that they are as low as is practicable. We aim to operate within the range of shock levels that well trained animals will risk receiving in order to have the chance of obtaining a very small amount of food. Our shock level are a maximum of 0.5mA for a maximum of 1 second however typically we would use a shock level between 0.05mA and 0.3mA and for 0.5 seconds. These shock levels are enough to produce a flinch or escape response. In comparison if a human was exposed to this level of electricity, they would not register any sensation at all. Animals will **typically** receive up to 15 of these mild shocks over a period of 3 days (with a maximum of 20 per experiment). The mice are able to escape these shocks by simply jumping. Animals will be monitored to ensure that the shock levels applied produce the appropriate flinch or escape responses (by analysis of the unconditioned response to the shock). For new genotypes/experimental manipulations we will check to ensure that our footshocks do not result in prolonged vocalisation. If prolonged vocalisations were observed then shock levels would be reduced. If we were to observe any animal continuing to suffer lasting pain/stress after the footshock procedure it would be humanely killed.

Learning is assessed both during the training sessions and in periodic extinction tests. This allows us to track the learning of the animals and avoid over-training.

The use of treadmill exercise tests can be a suitable independent or complementary alternative to wheel running allowing assessment of fatigue-like behaviour in a greater number of mice over a shorter time frame. This alternative also limits the need to use single housing of animals.

Why can’t you use animals that are less sentient?

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. We can't use embryos as their immune system is immature and the use of this model is for the most part not relevant for assessing tumour growth and treatment response in adults or children.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will follow published tumour model guidelines to ensure adequate pain relief is provided where necessary and severity is kept to a minimum. For pharmacological strategies to be tested, doses will be set based on our previous in vitro/organoid studies, or information from Industry/collaborators/literature in order to minimise the risk of toxicity. If necessary, tolerable doses will be first established and then subsequent doses will be

administered with the intention of reaching biologically active plasma levels. 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. To minimise recovery time when performing local irradiation, anaesthesia will be used wherever possible. Lead shielding will be used to cover the non-target field to minimise normal tissue damage to healthy tissues that are not being studied.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The following will be followed:

Guidelines for the welfare and use of animals in cancer research (BJC, 2010)
www.nc3rs.org.uk
https://norecopa.no https://www.lasa.co.uk

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have signed up to the NC3Rs website and will review the information published here regularly to check for new updates.

Also we will attend regular internal 3Rs meetings and will contact our NC3Rs regional manager for advice on how to implement relevant advances which could be applied to our work. We will also seek advice from our Named Information Officer.

60. Immune-Stromal interactions in infection, inflammation and cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Immunity, Stromal, Inflammation, Cancer, Infection

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

In this project we aim to understand how cellular communication between immune and supporting (stromal) cells occurs locally within tissues to provide protection in the context of infection, inflammation and cancer. Specifically, we want to understand how this happens within the space around the lungs (where cancers often grow and a parasitic worm can live), and the abdomen, and whether this is controlled by the interactions of immune cell clusters that are within specialised fat tissues within the chest and abdomen.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 defining the mechanisms underlying infection and cancer this work will enhance our knowledge of how immune and supporting cells interact and open new roads for the future development of therapeutic tools in the treatment of human disease

What outputs do you think you will see at the end of this project?

We will generate new data and publish peer reviewed impactful research articles in academic journals, obtain further funding from UKRI and charity funders. This project is primarily designed to address fundamental scientific questions however, the biological importance of the findings could be far reaching and is relevant to several aspects of human and animal health and disease.

Over ¼ of the human population and the majority of wild mammals are infected with helminths and trypanosomes, over the next five years we hope to understand better how the interactions of solid tissue and fluid phase immune cells interact to result in the local killing of tissue migrating parasites.

The pleural cavity is a site to which cancers of the breast and lung frequently metastasise, and ovarian cancers frequently metastasise to the peritoneal cavity and omentum. These cavities are also the sites at which asbestos and carbon nano-tube induced mesothelioma develop. We expect our work to uncover basic mechanistic details of how factors produced by immune cells and tissues within the serous cavities interact with invading carcinogens and tumorigenic cells promoting the development of malignancy.

Infection and inflammation within the lungs frequently results in activation of the pleural immune response and the development of pleurisy, a disease which is complicated to both diagnose and treat. Our investigations will provide vital information on the role of, and how interactions between, immune and stromal cells contribute to or limit pleural inflammation. We hope our studies will reveal new therapeutic targets to ameliorate the symptoms of this horrible disease.

Who or what will benefit from these outputs, and how?

We will produce primary research publications and communicate our research at local, national and international levels.

Short term: acquisition of knowledge using cutting edge scientific techniques to progress our understanding of immune activation

Medium term: sharing of novel findings will continue to support collaborative projects and funding applications with cancer biologists to facilitate continues cross-disciplinary studies to enable improved understanding of health and disease.

Long term: Identification of targets and development of therapeutic strategies to target effector cells during disease

How will you look to maximise the outputs of this work?

The findings will be disseminated to the wider research community at both the local (internal meetings within my research group and the microbes, pathogens and immunity divisional theme which I lead), national (at meetings such as the annual congress of the

British Society for Immunology and with established collaborators) and international level via presentation at scientific conferences and by publication in world leading scientific journals. New publications and resources will be promoted through our web pages and by social media where appropriate. Where negative results are viewed as useful to the community they will be published in open access formats.

Species and numbers of animals expected to be used

- Mice: 6250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 the proposed project, we will utilise sexually mature wild-type (non-genetically modified) mice, and mice modified to have alterations in immune cell function. Mice are known to have a large number of infection inducible fat-associated lymphoid clusters and a wide spectrum of genetically altered lines in which genes with immune function are depleted are available. Furthermore, mice are amenable to and tolerant of the proposed techniques within this project thereby increasing the reliability and reproducibility of data with least impact on pain and suffering.

Typically, what will be done to an animal used in your project?

Animals will be bred in captivity to over or under-express certain genes that are known to play roles in immune activation in diseases such as auto-immunity, cancer, obesity, pleurisy and infection. These genetic manipulations are known not to cause any noticeable harm to the animals in the normal, healthy state. The animals will either be infected with a species of helminth, or trypanosomes, or have inflammation induced via delivery of inflammatory agents such as a hydrocarbon oil, bacterial or fungal preparations, bio-persistent fibres or have cancerous cells injected directly into the pleural space, or via development of a solid tumour. The animals may subsequently be treated with substances that will modify the immune response. These animals may also have their diet manipulated, for example through changing the fat composition of their food or via modification of drinking water. The majority of the experiments will last for less than a month with many lasting only a few days to a week.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of animals will experience only mild adverse effects. Parasite infections and products mimicking parasite infection are generally well tolerated. In order to investigate the interactions of tumour cells, fungal allergens and inflammatory agents (including fibres like asbestos) with the pleural cavity, some animals will experience moderate pathological symptoms, in the form of inflammation in the lung & body cavities. Such inflammation does not cause the animals great amounts of distress, and any which appear generally ill (and reach a score of 5 on our scoring guidelines), or have difficulty breathing will be

immediately sacrificed and all animals will be killed before they exceed moderate severity limits. Some animals (<100 estimated over duration of the project) will develop a primary solid tumour of the mammary fat pad which will enable the characterisation of tumour cell metastasis to the lung and pleural cavity.

At the end of the experiment, all animals will be humanely culled.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- No animal will experience severe effects.
- 10-20% of animals may experience moderate severity. 40-50% will experience mild severity
- 30-40% will experience a sub-threshold response

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 adequately replicate the highly complex interactions of the immune system with local tissue environments, physiological processes (such as breathing) and insurgent pathogens, carcinogens and inflammatory molecules is not possible without the use of animal models.

Which non-animal alternatives did you consider for use in this project?

Utilisation of in vitro cell culture studies. Analysis of immune cells from patients.

In vitro systems will be utilised where possible, for example co-cultures of FALC containing tissues with tumour cells, parasites and inflammatory agents will be used for imaging purposes and to address the secretion of factors by the stromal, immune and adipose compartments of the pleural and peritoneal cavities. Macrophage activation by various cytokines, chemokines, tumour cell lines and growth factors will be carried out using both murine derived macrophage populations differentiated in vitro from bone marrow cells, enabling production of large numbers of macrophages, and using the THP-1 human monocyte cell line; resulting in the use of fewer mice.

Concurrent to the use of mouse models, we plan to continue our analyses of immune cells in pleural fluid from a cohort of patients with both benign, malignant and infectious pleural effusion, enabling a direct comparison between our models and that of clinical specimens.

Why were they not suitable?

Specifically, no alternatives exist for complex interactions of a complete immune system, nor parasite and tumour cell migration through the body, as such we cannot replicate these studies in vitro.

Throughout the duration of the project, further alternatives will be sought and resources such as the NC3Rs website will be periodically reviewed to ensure any suitable advances in the replacement of animals are employed in this programme of work.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used pilot data and previously published data to design our experiments. Experimental design will be reviewed periodically as new data is generated. Numbers are based on our experience of managing a project of a similar scope over the same duration.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used experience from previous studies and the NC3Rs experimental design assistant to determine the number of experimental animals required. In some instances, where such use is experimentally viable we intend to utilise delivery of cre-recombinase expressing vectors rather than inter-breeding of genetically modified cre-recombinase expressing animals to 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?

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 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; if the animals are as alike as possible the responses we measure are expected to be less variable. We have carefully calculated the minimum number of mice required for the experiments described in

this project to ensure that the findings generated via experimentation are not likely to occur randomly or by chance, but are instead likely to be attributable to a specific cause. We will consult statisticians whenever necessary to ensure this is the case.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 the proposed project, we will utilise genetically modified mouse strains deficient in molecules involved in the function of specific immune cell subsets. Mice are known to have a large number of infection inducible fat-associated lymphoid clusters and a wide spectrum of genetically altered lines in which genes with immune function are depleted are available. Furthermore, mice are amenable to and tolerant of the proposed techniques within this project thereby increasing the reliability and reproducibility of data with least impact on pain and suffering.

Throughout, we will ensure that the least invasive methods of dosing and sampling are applied, including the use of anaesthesia for humane restraint when appropriate. Analgesia will be used in all situations that warrant such, for example prior to tail vein sampling.

All immune-compromised mice (GA strains lacking specific immune cell populations) will be housed in IVC cages to reduce risk of infections by opportunistic pathogens.

Animals under procedure will be observed daily at the point where symptoms are most likely to develop. Humane endpoints will be determined using a scoring system based on disease load and clinical observations (hunching, piloerection, grimace, normal movement, body temperature, weight) and will be adhered to at all times to minimise harm.

Delivery of avirulent Newcastle disease virus expressing defined pathogen associated proteins is a refinement to minimise animal suffering while enabling greater understanding of the immune response to virulent pathogens to be understood.

Intra-pleural injection to model early seeding of the pleural space to complement induction of solid tumour formation followed by subsequent metastasis will reduce the number of animals that undergo solid tumour development. Minimal tumour cell numbers will be injected directly into the pleural space to model metastatic seeding of the cavity, negating the requirement for a solid primary tumour.

We will regularly check information on the NC3Rs website and continue to utilise the PREPARE guidelines to ensure experiments are conducted in the most refined way.

Why can’t you use animals that are less sentient?

Although lower organisms (Drosophila/Zebrafish) do have primitive cellular systems, these do not have the complexity of mammalian systems. Mice have comparably complex immune cell populations to those of the human within the body locations we are interested in better understanding. Colonies of mice exist that possess defined changes within their DNA resulting in specific modification of immune cell function and a wealth of reagents are available for the analysis of the mouse immune system, as such, mice provide an unrivalled system to answer the questions posed in this project. Furthermore, no alternatives exist for modelling parasite or tumour cell migration through the body, as such we cannot replicate these studies without using a mammalian model. Whenever possible we will use cell culture systems to address defined questions.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Throughout, we will ensure that the least invasive methods of dosing and sampling are applied, including the use of anaesthesia for humane restraint when appropriate. Pain relief will be used in all situations that warrant such, for example before collecting blood from the tail.

All mice will be housed in individually ventilated cages to reduce risk of infections by opportunistic pathogens.

Animals under procedure will be closely observed, clinical signs (including hunching, grimace, normal movement, body temperature) will be monitored using a scoring system and used 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 adhere to the PREPARE guidelines to ensure that the experimental design of our studies, the communication between scientists and animal technicians and the control of our experimental procedures is the most refined possible (Smith et al., 2018).

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 continue to utilise the PREPARE guidelines to ensure experiments are conducted in the most refined way.

61. Modulating Wound Healing for Therapeutic Applications

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Chronic Wounds, Skin, Infection, Treatment, Therapy

Animal types	Life stages
Mice	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to assess the effectiveness of potential new and existing treatments for chronic non-healing wounds. This research will support the development of new therapies to promote healing in the elderly and diabetic.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chronic, non-healing wounds (including venous ulcers, pressure sores and diabetic foot ulcers) are a major problem for the elderly and diabetic. Poor wound healing causes

extensive psychological and physical suffering to patients, often leading to severe amputations and loss of life. Unfortunately, the number of people affected by chronic wounds continues to rise due to the expanding elderly and diabetic populations. By 2025, 5.4 million people in the UK will be diabetic, and one in five people will be over the age of 65. It is therefore not surprising that non-healing wounds are also a huge financial burden, currently costing the NHS up to a staggering £8 billion each year to treat.

Many non-healing wounds persist for months to years, preventing patients from living normal lives. Yet poor healing remains an entirely underappreciated area, receiving considerably less investment than other areas of research (such as cancer and heart disease). Current treatments often do not work because their effectiveness is rarely assessed using suitable laboratory models. The work outlined in this project is specifically designed to address this gap in knowledge, supporting the testing and development of more effective treatments for human chronic wounds. In addition, there are broad similarities between wound healing and cancer, meaning that treatments evaluated in this licence could also be useful for the treatment of cancer.

What outputs do you think you will see at the end of this project?

This work will support the development and testing of new and existing therapies to promote wound healing. This is important as non-healing wounds cause a substantial physical, social, psychological and economic burden. However, there are currently no universally effective treatments for non-healing wounds, and current treatments often lack suitable experimental evidence of effectiveness. As a result, many patients face considerable stress, life-changing amputations, or loss of life entirely.

Patient Benefits

We will contribute to patient benefit by performing pre-clinical efficacy testing studies of new and existing wound treatments. Where applicable, we will first validate effectiveness of therapies in laboratory human cell and skin models, before confirming their efficacy using delayed healing mouse models (e.g. aged and diabetic). These experiments will allow us to determine the effectiveness of potential treatments and will inform how these treatments should be administered in follow-on clinical studies. We already have strong links with clinical colleagues to be able to set up these follow-on studies, and we are currently involved in a number of clinical trials. Overall, this work should result in the clinical implementation of safer, more effective treatments for non-healing wounds in humans.

Commercial Benefits

Pre-clinical efficacy testing will have several commercial benefits:

Assessment of product efficacy during early-stage production will de-risk follow-on steps of the development pipeline.

Pre-clinical safety and efficacy data is an important step of the regulatory process. Pre-clinical data will help to identify the conditions (e.g. dose, duration, method of delivery) that will be most likely to work in the clinic.

Mode-of-action data for existing products will allow industry partners to understand how

their products work in-detail, which will provide them with an advantage over competitors, give buyers/clinicians confidence in their products and ultimately increase sales.

Access to widely validated pre-clinical models will enable commercial partners to continue to improve their existing products.

Academic Benefits

The work outlined in this project licence will enable us to explore the mode-of-action of a range of new and existing therapies for chronic wounds by using clinically relevant models of healing (e.g. aged and diabetic mice). This level of detailed information is often lacking for current treatments, where the only output required from a regulatory perspective is time to full wound closure. Therefore, new data demonstrating how these products work will be novel and highly publishable, and of interest to a wide range of researchers in academic, clinical and commercial settings. Publication of this work will provide a platform for the field, informing clinical treatment strategies, and promoting the implementation of more rigorous efficacy testing.

We will combine our animal research data with our findings in human skin models, which will contribute to a number of high impact publications in field-relevant journals, and dissemination of findings at national and international conferences. This approach will maximise the likelihood that the outputs from this project will lead to follow-on funding and first-in-man clinical studies. In addition, there will be opportunities to patent our work and generate multiple streams of income to continue our research.

Who or what will benefit from these outputs, and how?

In the short-term (1-3 years), this work will have academic and commercial benefit by providing proof- of-concept data that will be highly publishable, patentable and accelerate commercial route to market. For existing products, our mode-of-action data will support claims, increase sales and lead to greater implementation of highly effective treatments in the clinic (which will provide short-term benefit to patients). Moreover, there will be opportunities to apply for joint academic-industry funding with commercial partners to support future development opportunities.

In the mid-term (3-5 years) development and validation of novel therapies in pre-clinical studies will provide the safety and efficacy data required for early-stage clinical trials.

In the long-term (5+ years) we will expect to see wider patient benefit from novel therapies if clinical trials prove effective, and we would develop our platform as an initiative for more rigorous testing of existing wound treatments.

How will you look to maximise the outputs of this work?

Outputs of this project will be maximised as follows:

We will meet often to discuss our findings with our collaborators (academic, clinical and commercial) to determine follow-on steps to accelerate our outputs for patient benefit.

Where appropriate, research findings will be disseminated across multiple sectors through conferences, publications, outreach events, patient support groups and via our

communications teams.

We will combine commercial outputs with academic methods to provide resources for the wider research community (e.g. publishing optimised protocols and big data sets). This will significantly enhance the quality of pre-clinical data produced and will encourage a step-change in current commercial wound research approaches, where therapeutic evaluation is limited.

Species and numbers of animals expected to be used

- Mice: 2160

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mammalian wound healing is a complex process that involves multiple overlapping stages and interactions between a wide range of cell types and environments. The wound healing process has evolved over millions of years to allow us to heal our injuries quickly and efficiently. Lower organisms (e.g. flies and frogs) lack some of this complexity and have very different skin structures to humans. For our work to translate to the clinic to benefit patients, we need to use models that better recapitulate the human wound healing response. The most widely used models that mimic human wound healing more closely are mice, rats and pigs. These animals go through the same wound healing stages as humans (including inflammation, reepithelialisation, angiogenesis and granulation tissue formation). There are some differences in skin structure, with pig skin most closely resembling human. Pig skin also undergoes less contraction than mice and rats.

We use mice because they are the least sentient of the appropriate models and the easiest to work with, while still giving the same useful outputs. There are also widely published and validated mouse models of poor wound healing, such as ageing, diabetes and infection. As our major goal is to test products for their clinical efficacy, the use of these models will provide important information that will improve the likelihood of treatments being effective in patients. We also require adult mice because the immune system and skin of young mice is not yet fully developed. Indeed, wound healing in underdeveloped skin is completely different to adult skin and will therefore not provide clinically meaningful data.Some groups use a splinted mouse wound model to reduce contraction, however, in our experience this is not necessary to achieve the desired outputs.

Typically, what will be done to an animal used in your project?

An adult mouse will be bought in from a registered supplier and acclimatised to the environment. For the experiment, the mouse will be given general anaesthesia to fall asleep and will be placed on a heated pad to keep it warm. The back of the mouse will then be shaved with clippers and the area prepared for wounding. While the mouse is still asleep, two small skin cuts will be made, and pain relief will be given via an injection to

prevent any short-term pain when the mouse wakes up. While the mouse is still anaesthetised, we will add test treatments to the wounds, which could be creams, liquids and/or dressings. The mouse will then be moved to a heated, ventilated area to recover and will be given a clean cage with fresh bedding, food and water. The whole procedure will be brief, and the mouse will wake up quickly following the procedure. The mouse may then be put under very brief general anaesthetic every few days to add fresh treatments and/or take images of the wounds as they heal. The mouse will not undergo any other procedures. At the end of the experiment, the mouse will be culled painlessly, and the wounds will be collected for laboratory analysis to assess the efficacy of treatments.

What are the expected impacts and/or adverse effects for the animals during your project?

There are no expected adverse effects from the procedures performed other than potential mild and transient pain. Some animals may lose a small amount of weight initially, but generally recover well from the procedure. All animals are expected to only experience transient pain from their small skin wounds, and they will be given pain relief at the time of surgery and wherever necessary.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Over 90% of mice will have a moderate experience of the wounding procedure.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The wound healing process has evolved over millions of years to rapidly repair damage following injury. It is an immensely complex process involving interactions between circulating immune cells, local tissue cells, signalling molecules and the matrix that surrounds cells. Skin is also a highly complex tissue which includes blood vessels, nerves, hair follicles, sweat glands, sebaceous glands and other appendages. There are currently no laboratory-based cell (*in vitro*) or tissue (*ex vivo*) models that come close to fully replicating the complexity of the wound healing process within the body.

A number of animal models are available that heal wounds in a broadly similar manner to humans. The main models used are pigs and rodents, but in this study, we will use the least sentient model that can still provide clinically meaningful information (mice). There is extensive published literature demonstrating that:

Mouse skin structure broadly resembles human skin structure.

The main processes that are involved in tissue repair (inflammation, proliferation, and matrix remodelling) are all found in mouse wound healing.

There are well validated models of delayed wound healing, such as aged and diabetic mice. These mice mimic the diabetic and ageing process in humans, and possess wounds that heal poorly, like human chronic wounds. As above, there are no suitable *in vitro* or *ex vivo* models of ageing or diabetes, which affect the whole body.

The ultimate goal of our research is to assess the effectiveness of treatments for the clinic. *In vivo* safety and efficacy data is currently a pre-requisite for obtaining ethical approval for first-in-man clinical studies, while using suitable models will enhance the likelihood a treatment will translate to patients.

Which non-animal alternatives did you consider for use in this project?

We routinely use a range of established *in vitro* and *ex vivo* models alongside *in vivo* studies to comprehensively evaluate the wound healing process and assess the efficacy of potential treatments. This is also essential to maximise the likelihood that effects shown in mice will also translate to the clinic. The assays that are available complement, but cannot currently fully replace, *in vivo* studies.

However, they can reduce the number of animals needed for certain experiments.

***In Vitro* Assays**
We routinely use a wide range of *in vitro* wound healing assays where possible. These include scratch wound migration assays with different types of human skin cells, assays using human immune cells (e.g. to measure their ability to kill bacteria), assays to measure blood vessel formation, and assays that measure the growth of cells and their ability to make collagen and other skin proteins.

Co-Culture

To better model the complexity of actual wounds, it is possible to grow different cell types together. These models include growing two different cell types in close proximity so they can share secreted factors and communicate with each other (co-culture).

Skin Equivalents

Building upon the concept of co-culture, it is possible to grow very simple skin-like structures in the laboratory that contain some of the cell types found in skin. These are known as skin equivalents. They can be wounded and will undergo some of the processes observed during *in vivo* wound healing but they still lack many important components of actual skin and the wound healing response (such as inflammation and skin appendages).

Ex Vivo Wounding

A relatively new model for skin and wound studies involves culturing living human skin in the laboratory for up to seven days. This skin can be obtained as surplus material following

surgical procedures. *Ex vivo* skin can be wounded and will heal to a certain extent outside the body. But again, it lacks several of the key processes involved in wound healing (e.g. a circulating immune cell response).

Pathological Wound Healing

Each of the above models can be modified to mimic poor healing, for example by using cells and tissues from aged and diabetic individuals, or by culturing cells in high glucose to mimic the diabetic environment. However, progress in this area is extremely limited and no models fully replicate ageing and diabetes in the body.

Why were they not suitable?

We routinely use almost all of the above non-animal models where possible. For example, we pre- screen drug compounds using *in vitro* and *ex vivo* models before moving to *in vivo* studies. However, it is widely acknowledged that none of the available models are currently able to fully replace *in vivo* studies. There are broad limitations that apply to all of the non-animal models. These include:

The inability to evaluate whole body effects of treatments on wound healing. This is important to evaluate whether a potential treatment has off-target effects (i.e. an unwanted/unexpected effect on another part of the body).

The inability to give drugs via different administration routes to understand pharmacological uptake.
The short nature of non-animal models. Human wound healing can take up to two years, while these models generally only last up to seven days.

The limited translational relevance of non-animal models, for example when considering ageing and diabetes. Ageing and diabetes change cells throughout the entire body, altering the way they interact with each other and their environment. These interactions are currently far too complex to mimic in currently available non-animal models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We estimate to use up to 2160 mice in our wounding protocol over the course of the five-year licence. This will be dependent on the number of funded studies that are secured. However, from experience, it is estimated that we would undertake one study every month, with an estimated 36 mice used per study. This equates to up to 432 mice per year.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Literature searches will be performed to determine the safety of proposed drug treatments for use in animal experiments, and their safe dosages where available. Potential treatments will first be screened by using *in vitro* and *ex vivo* laboratory models and will be dependent on the type of treatment assessed. This will allow us to screen a range of drugs/treatments at different concentrations and using different dosing strategies to find the most effective treatments and optimal dosing regiments for use during *in vivo* experiments. Using this pre-screening technique in non-animal models will significantly reduce the number of mice required for *in vivo* wounding experiments. This approach will not only demonstrate proof of efficacy prior to undertaking *in vivo* studies, but will also increase the likelihood that observed *in vivo* effects will translate into human clinical studies (where human-based laboratory studies have been undertaken).

We have consulted the NC3R's Experimental Design Assistant in planning our studies, along with the PREPARE guidelines. In all experiments, standardised protocols will be used including all relevant control groups, treatment randomisation with each mouse being an experimental unit, time-matching of control and experimental groups, and matching for gender, age and weight where necessary. We will not use sham surgery controls. Data from pilot studies will be included as experimental data where possible to reduce the number of mice used in subsequent studies. For example, if a pilot study using 3 mice per group showed that 8 mice per group were required to gain significant findings, the follow-on study would only use 5 mice per group and would be combined with the pilot study to give 8 mice per group. We will ensure that numbers of mice used are correct, and that we are using the minimum number or animals required to gain statistical significance.

All subsequent analysis will be carried out blinded so that researchers do not know what groups each sample was placed in. Interventions and outcome measures will be optimised to validate the study hypothesis with the minimum number of animals. Where applicable to the study outcomes, we will photograph wounds throughout an experiment to assess healing over time and we will only collect mice at specific time points post-injury. By photographing wounds and only collecting mice at one or two healing time-points, we will significantly reduce the number of mice that we will use (as we will not need to collect animals each day/every few days). We will collect mice at time-points post-injury that will be the most informative based on our extensive experience of the murine wound healing response. In addition, we follow a standard protocol for tissue collection and use established laboratory procedures.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Extensive literature searches will be carried out in order to avoid the unnecessary repetition of experiments where appropriate. However, the majority of the proposed studies will involve testing of novel products. The work of others in the field will be constantly reviewed by the research team and disseminated to the academic-commercial research team. We will reduce experimental variation significantly via extensive training of investigators, the use of optimised techniques, and by reducing compounding factors such as age, genetic background and hair cycle stage.

We will use *in vitro* and *ex vivo* pilot studies to optimise experimental treatment conditions prior to undertaking *in vivo* work. Where possible we will maximise the use of tissue obtained from experimental animals, sharing with other groups as appropriate. Additionally,

our institution routinely sends out a tissue sharing list to allow other researchers to make use of any surplus animal tissue.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice exclusively as they are the least sentient species that are appropriate to address the aim of this project. We will use highly validated and optimised dorsal incisional and excisional acute wounds (small injuries to the skin on the back). We have chosen these wounding methods as they cause minimal discomfort compared to other models (e.g. burns, splints and pressure ulcers) but still allow us to address the aim of this work. There is an upper limit to the size of the wounds we will use to minimise the amount of inflammation and pain to each animal. In addition, mice will be asleep for the procedure and will be given pain relief to reduce any post-surgery pain. From experience, pain experienced will be short-lived and mild and mice will make a rapid and full recovery, returning to normal behaviours within minutes (e.g. grooming and eating).

Mice will be singly housed after the procedure but only for the minimal time needed to ensure early wound healing is not affected by mouse interaction (maximum two weeks). Treatments will always be administered by the least invasive appropriate method, often topically applied to the wound.

Why can’t you use animals that are less sentient?

We will use mice exclusively as these are the least sentient species that are appropriate to address the aim of this project. Other models are available ranging from embryos and developmental models (flies and frogs) through to large animals (pigs). A main focus of this project is to assess the efficacy of commercial products designed for use in the clinical setting. As such, embryonic and developmental models, while less sentient than mice, are not appropriate as they cannot model the human aetiologies of poor healing (ageing and diabetes). While zebrafish may be a less sentient and useful model for laboratory research, there are a number of reasons why they are not a good model for human wound healing. Zebrafish skin structure is significantly different to human skin. The epidermis of the zebrafish is also covered with scales and lacks the hair follicles and glands present in the skin of mammals. In addition, wound healing stages in zebrafish occur in isolation, and do not overlap like in mice and humans. Another difference is that zebrafish heal with little scarring, while in humans, scarless healing only occurs under very specific circumstances (e.g. in the mouth and during fetal development). As a result, the mouse has been selected as the least sentient species available to adequately assess poor healing in mammals. Terminal anaesthesia is inappropriate for this study as we will be assessing wound healing over a time frame of days and weeks and cannot keep the animals under

general anaesthetic for this amount of time as it is harmful and not practical.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Post-operative monitoring will be performed following each procedure, with timings and frequency depending on the procedure performed. Animals will then be monitored at least daily for signs of distress with observations recorded for each animal so that potential deviations will be realised quickly. Following surgery, animals will be placed in a warm, ventilated cabinet and monitored until they have recovered. The animals will then be placed in a clean cage with bedding, food and water. Analgesia will be administered as required to alleviate short-term discomfort to the animals. Extensive training will be provided to researchers undertaking experiments to ensure standardisation of the wounding technique and to ensure awareness of animal welfare and humane endpoints. Any treatments will be administered using the least invasive method to reduce harm to the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the PREPARE guidelines to design and implement experiments in the most refined way. There is no official best practice guidance for wound studies per se, but there are a number of recently published methodological reviews (e.g. Rhea and Dunnwald, 2020, *J Vis Exp*, 162:e61616) that we will use to establish standard operating procedures and ensure best practice. We will follow LASA guidance on asepsis for all experimental studies. We will continuously review our protocols and procedures in line with updates to the PREPARE guidelines and published literature. We will also discuss planned strategies with the wider research team (academic/clinical/commercial).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our group is at the forefront of wound healing research, we regularly attend international conferences, and are part of a large network of *in vivo* researchers. We will carry out extensive literature searches to ensure this project avoids the unnecessary repetition of experiments and we will keep up-to-date with the scientific literature. We will also have regular team meetings to discuss project updates, while relevant developments will be disseminated with industry experts and collaborators.

We will always adhere to the ARRIVE Guidelines 2.0 (du Sert et al., 2020, *Plos Biology*, 18(7):e3000411) when publishing and the NC3Rs EDA tool and PREPARE guidelines when designing and implementing experiments (Smith et al., 2017, *Lab Animal*, 22:135-141). We will utilise our extensive knowledge and previous experimental data to ensure experiments utilise the least number of animals and are as refined as possible, and we will continue to revise our approach as new data emerges. We subscribe to the NC3R's, FRAME and Animal Free Research UK newsletters and keep abreast of topical advances in 3Rs research and policy. To ensure that the research team are kept up- to-date with the latest developments in the 3Rs, we will hold regular project licence meetings, attend institutional seminars and review pertinent case studies.

62. Neuroinflammation as a driver of neurological 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

Immune therapies, microglia, white matter diseases, brain imaging, zebrafish

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, 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 understand how our immune system contributes to the development of brain diseases and how to manipulate it to develop 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?

Leukodystrophies (literally, from the Greek, leuko - white; dystrophy - imperfect growth) are inherited brain disorders characterised by lesions in the brain that result in severe mental and physical disabilities, mostly in children. Often fatal, there are thought to be over 200 forms of leukodystrophies, affecting an estimated 31 cases per million live births. Treatment options are limited, partly due to the lack of animal models that reproduce the

clinical sign observed in patients. For example, rodent models of leukodystrophies often fail to show brain lesions. We will use zebrafish models of leukodystrophies that reproduce symptoms seen in humans in order to better understand the basis of these diseases and to develop ways to treat them.

What outputs do you think you will see at the end of this project?

Developing and analysing a zebrafish model of leukodystrophy will provide a better understanding of the molecular and cellular mechanisms of leukodystrophies. Our experiments will also provide new knowledge about the types of cells and the genes that cause inflammation of the brain.

The expected outputs and benefits of our work are:

A greater understanding of the type of inflammatory response that causes neurological disorders.

Identification of factors that trigger inflammation of the brain: these factors could then be targetted genetically or pharmacologically as novel therapies.

Establishing zebrafish models for testing therapies to treat leukodystrophy: drug treatment, gene therapy and cell therapy. This is a worthwhile and long-term benefit as there is no current therapy for patients. We currently have 3 established models and plannign to develop another 2 models of common forms of leukodystrophy.

Who or what will benefit from these outputs, and how?

The beneficiaries from this project will be:

Patients and their families (Short and Long term): I regularly attend patient meetings to communicate our research efforts using zebrafish models. This has been enthusiastically received by Leukodystrophy families, as our research provides hope to know ongoing research efforts are happening for their disease. Findings from our zebrafish models have been used to guide and inform more clinical studies; I plan to continue work that will benefit these patients, their families and work closely with clinical experts. Longer term, more translational research to further develop and test new therapies should be possible.

Zebrafish researchers (Short term): We are developing new methodologies to study immune cells and the zebrafish brain, methods that will be communicated at scientific conferences and through publications so they can be adopted by other researchers.

Wider clinical and research community (Short and Long term): Our work using zebrafish will contribute to the understanding of rare human diseases. Our model can also help to identify new drugs for therapeutic development. As part of the leukodystrophy research network I developed, I will create robust partnership with the pharmaceutical sector to drive our research to clinical application.

How will you look to maximise the outputs of this work?

The data generated by this programme of work will be communicated to the scientific community via publications in international peer-reviewed journals, as well as via presentation at national and international conferences.

To increase the impact of our work, I will communicate our findings through the UK leukodystrophy network (www.leukolabs.org), reaching out to clinicians, patients and industry. I will continue to attend annual family meetings and outreach events to present to affected families.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 29708

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 good model for human disease because they are genetically similar to humans, rapidly developing and easy to breed. Their transparent embryos allow for non-invasive imaging and they are small and inexpensive to maintain. They are also anatomically similar to humans because they are vertebrates with a spinal cord along their back and are good for studying neurological disorders. By creating zebrafish models with mutations in genes that are known to be involved in neurological disorders, we can study the effects of these mutations on brain development and function. Creating disease models in zebrafish will be useful in the development of new drugs for neurological disorders.

Zebrafish are the right model to study leukodystrophy because they are transparent at the larval life stage, when we study brain formation and behaviour of immune cells. Visualising brain development will be very relevant to understand leukodystrophies that start in very early years. Brain cells and immune cells are the same in both zebrafish larvae and humans: this means what we learn in zebrafish is very likely to be applicable in humans. Importantly, zebrafish can be genetically modified to create models for new diseases, for instance by disrupting genes that are known to cause leukodystrophies in patients. Larvae are small, so drugs can be tested on larvae in petri dishes (as you can with cultures cells) however the zebrafish larvae have a complete, complex nervous system and they also provide the context and benefits of whole-organism investigation.

Typically, what will be done to an animal used in your project?

Creating models of leukodystrophy: We will create mutations in zebrafish embryos, raise them and breed them to create genetic lines with the same defect as in leukodystrophy patients.

Experiments on zebrafish larvae: Most often experiments will be carried out on animals before they become protected under UK legislation (<5 days post fertilisation); pre-feeding larvae. We will characterise the effect of the mutations in the genetic lines created in (i) on

brain development and immune responses. We will use high resolution microscopes to create detailed images of the transparent larval brain, as well as carry out gene expression and biochemical analysis.

Experiments on adult fish: Zebrafish mutants that reproduce human symptoms (such as swimming defects that mimic physical disabilities in patients) will be studied as older larvae and adults using imaging of the brain using similar techniques to what is used in humans (magnetic resonance imaging; MRI). Therapies, such a cell transplantation, and drug treatment will be tested to improve the clinical signs.

Adult animals will be kept for up to 30 months, as long as they remain fertile and healthy.

What are the expected impacts and/or adverse effects for the animals during your project?

Gene mutations that cause brain disease can have adverse effects which mainly includes physical disabilities that gradually appear in the way the fish swim over a few months. They start by swimming slightly to their side, after a few weeks this can worsen with fish swimming on their side with their body fully tilted. At this stage, fish can still control their movement, reach food, and swim with the rest of the group. However, after a few more weeks, these fish will start losing control of their movement and start spiral-shape swimming. At this point, animals cannot feed effectively and are humanely killed.

Rarely, zebrafish mutant for brain disease can have weakened blood vessels and suffer haemorrhages, as is seen in human patients with small bleeds in their brain. Seizures, which are very short bursts of muscle contractions, can also happen in neurological disease fish models.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

54% of animals will be used for breeding and maintaining our stocks of zebrafish and will therefore experience sub thereshold severity.

The rest of the animals are under moderate protocol, under which only a small percentage will experience this severity. Due to the genetic nature of the disease, in most cases only 25% of those animals will develop moderate severity and the rest will be 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?

Brain formation and inflammation are complex processes requiring interaction of multiple cell types and three-dimensional tissue changes. This cannot meaningfully be modelled in vitro.

Which non-animal alternatives did you consider for use in this project?

We have developed in vitro models, such as mouse cell lines, to study how a mutation can affect the early stages of embryonic development. We also have collaboration in place with human stem cell laboratories, to study leukodystrophy-causing genes in cultured immune and brain cells in vitro.

Why were they not suitable?

In vitro models can validate some of the results we are generating in fish, but they will never be as complex as a brain. We will use in vitro models for very specific questions that are related to the specific cell type cultured, for example interaction between immune and different nerve brain 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?

Most experiments are performed on larval zebrafish. Therefore, we have planned to keep sufficient numbers of adult fish to ensure a steady supply of embryos for our experimental work. The numbers of adults are under constant review to ensure that they meet this demand but not exceed it. The mature fish in this programme will be used multiple times to assess brain integrity using live brain scans throughout their life. This will avoid having to kill a fish each time we want to assess their brain.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used published datasets to predict groups sizes needed to generate data which will produce statistically relevant outcomes. Where appropriate we will use the NC3Rs Experimental Design Assistant to consult on sample sizes for individual experiments to ensure only the minimum number of animals required is used. We have performed pilot experiments in order to ascertain key timepoints.
We have searched on-line databases to identify 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?

Zebrafish breed very well, so we are able to obtain large numbers of embryos from a small number of parents. To minimise animal use, once experiments have been performed, sperm will be frozen (for future IVF) and stocks of adult animals will not be maintained. Pilot studies are performed initially to inform on the optimum number of animals needed per experiment to see a significant effect. Spare larvae will be used for other experiments or shared with other members of the laboratory for 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.

This project will use zebrafish in larvae and adult forms. We will use genetically modified animals to model human brain disorders which will cause similar symptoms to what the patients experience: difficulty to move being the primary symptom. The methods used will aim to study those symptoms while minimising animal distress and reduce those symptoms using therapeutic treatments.

Why can’t you use animals that are less sentient?

Zebrafish are the model with the lowest neurophysiological sensitivity suitable to study a whole brain.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Most animals will be under a mild severity protocol. Some animals will be under moderate severity to accommodate for swimming and physical deficits that some neurological mutant might develop. Our therapeutic regimes will attempt to reduce those moderate symptoms for the fish to return to full health. We will aim to refine experimental design wherever possible and are routinely using anaesthesia to minimise harms.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For all of our studies we will abide by published guidelines from the NC3Rs, as well as local guidelines, and ensure best working practice. We will also adhere to the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines) and use resources particularly suited to fish work such as the Prepare Guidelines from Norecopa (https://norecopa.no/prepare).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I participate and present at many workshops (LASA meeting, NC3Rs workshops) on animal usage in research and experimental design where I keep myself updated with the best practice in zebrafish research to constantly improve our protocols.

Additionally, I attend scientific meetings and discuss welfare and best practices with my broad international network of zebrafish users. Our facility has user meetings where 3Rs practices are introduced to licence holders to ensure best practices are used.

63. Evaluation of Vaccine Candidates for Group A Streptococcus Infection

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Group A Streptococcus (Group A Strep, GAS), Vaccine, Immune response, carbohydrate

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim is to identify novel vaccine candidates for the treatment of Group A Streptococcus. We will conduct a step-wise approach, first validating vaccine safety and efficiency to stimulate an immune response, followed by testing the protection provided by the vaccine candidates in mouse models for GAS infection (nasal and soft tissue models). These infection models represent the most common naturally occurring infection in humans with >700 million cases / year.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Our research addresses the prevention of human infections caused by the human-exclusive pathogen Group A Streptococcus (GAS). GAS causes a range of mild and severe diseases such as scarlet fever and streptococcal toxic shock, with more than 500,000 deaths annually worldwide. The effectiveness of current treatments for some infections is waning because of antibiotic resistance and the emergence of hypervirulent strains (those that can adapt to cause organ-invasive serious infections). In 2013 there were more than 1400 cases reported of invasive GAS infection in the UK alone, with mortality rate of > 30% within 30 days.

To reduce infection in humans and animals, so-called vaccines are given, that allow our immune system to develop antibodies against the pathogen. This route called vaccination has greatly benefited humans during the COVID19 pandemic. Many vaccines exists for the prevention of common disease including flu and hepatitis, however, to date, no vaccine has been successfully developed against GAS. This is due to the high number of GAS variants (serotypes), meaning that there are too many targets to generate a specific vaccine. However, a molecule that is common to most of these variants has now been identified and we wish to explore the possibility that it will be a good target for vaccination.

What outputs do you think you will see at the end of this project?

The expected outputs from this study are one or two vaccine candidates validated to reduce Group A Streptococcus infection in mice. The findings from this study will be published after peer review in an international (open access) journal.

The proposed studies are part of our vaccine pathway:

1) preclinical trials showing a good, reproducible antibody response with a clear reduction in infection in two separate infection types; nasal infection model and soft tissue infection model

2) Approved vaccine candidates will enter safety and quality control studies including tests in humans.

Who or what will benefit from these outputs, and how?

In the short term, these outputs will benefit justification to conduct subsequent research studies (preclinical and clinical trials). The studies will obtain knowledge of vaccine candidate effectiveness of reducing the bacterial burden when mice are infected with Group A Streptococcus bacteria. In the long term, these vaccine candidates will have a potential benefit to the patients, preventing infections in humans. Should the proposed work lead to the development of one or more more vaccine candidates, e.g., safety and effective prevention and eradication of bacteria in the animal model, these vaccine candidates will be tested in humans clinical trials.

How will you look to maximise the outputs of this work?

We have established collaborations with experts in this area of research, who are also co-applicants and collaborators on the 5-year grant. The main outputs of the studies will be peer reviewed publications in scientific journals and attendance at national and international conferences.

Species and numbers of animals expected to be used

- Mice: 1600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Because of the very significant similarities in the immune systems of mice and humans, the mouse is a good model in which to test if the vaccine candidates can reduce infection. There are also excellent reagents and procedures in place in order to test the immune response in the mouse. We will use adult mice from around 10 weeks old, when the immune system is fully mature.

Typically, what will be done to an animal used in your project?

Typically mice will be injected with a vaccine candidate, together with a suitable adjuvant (a substance intended to ensure a robust immune response) and boosted at intervals thereafter, much as human vaccinations are performed. Blood samples will be taken at intervals to monitor antibody levels and other indicators of an immune response to the vaccine over a 12 week period.

Vaccine candidates that show a positive immune response will be then investigated for their ability to protect mice from GAS infections. Mice will be infected by dropping a small drop with GAS bacteria into the nostrils, a similar way humans are exposed to infection in the air. The level of infection will be measured by gentle wiping of the end of the nose on a dish that allows the growth of the bacteria over a 3 day period. Alternatively, mice will be injected with bacteria into the thigh muscle, and bacterial infection will be monitored for signs of soft tissue infection, such as swelling, redness, and weight loss over a 3 day period. Tail bleed samples might be taken to investigate spreading of infection into the blood. Mice will be killed humanely at the end of the experiment and bacteria counts measured.

What are the expected impacts and/or adverse effects for the animals during your project?

Under previous licence authority, we optimised the make up of the adjuvant and vaccine candidates to avoid adverse effects. We do not anticipate any side effects from the vaccine candidates. Following administration of GAS to the nose or injected into muscle, weight loss is expected, therefore, mice will be weighed prior to and following infection on a daily basis. A scoring system will be used to assess weight loss, coat condition, posture and level of activity. Additional bedding and wet food may be given to symptomatic mice.

If any observed symptoms persist for more than 24 hours or exceed the applied severity limit (see below), in discussion with trained staff, the mouse will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We propose three different protocols.

1) Vaccination only - We expect the severity experienced by all the animals in this study to be mild.

2) Respiratory infection - These animals will be exposed to the bacteria via the nose and will display clinical signs, unless the vaccine candidates are highly successful and provide immediate and efficient protection. Therefore, we expect the severity experienced by all the animals in this study to be "moderate". If the mice experience a "moderate" level of severity, it means that they do get sick, but they don't get extremely ill. They might show some signs of sickness, such as being a bit lethargic, or perhaps showing some other mild symptoms. However, the term "moderate" implies that the mice are not severely affected by the infection. They are still able to move around, eat, and perform basic mouse activities, even though they are not feeling their best.

3) Soft tissue infection - As for the respiratory infection model, these animals will be exposed to the bacteria via an injection through the skin to the thigh muscle. Lameness is the most likely expected adverse effect. We expect also a "moderate" level of severity, unless the vaccine candidates are extremely good to fight off the infection immediately.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In order to develop and test vaccine candidates, we need to administer to an intact immune system, which cannot yet be modelled satisfactorily without using a living animal.

Which non-animal alternatives did you consider for use in this project?

1) Reconstituting the immune system in sufficient detail and complexity in a cell culture dish and testing vaccine candidates in this model;

2) Using human volunteers to test our vaccine candidates against the bacteria that naturally colonise and infect only humans.

Why were they not suitable?

The immune system cannot yet be reconstituted in a cell culture dish in sufficient complexity to produce the types of responses seen in intact animals and humans. For example, we cannot "input" a vaccine in a cell culture system and measure antibody production as an "output" of the immune response. Direct human trials will not be feasible until there is sufficient evidence of safety and the ability of the vaccine candidates to induce the desired response in animal models. Human challenge studies with GAS bacteria (one serotype only) have now been reported and might be provide an important alternative in the next 10-15 years.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 immune responses, we have determined samples sizes of 10-12 per group based on preliminary data.

For infection models, we will perform a number of small pilot studies to validate the model at the establishment. Once established, group sizes of 10-15 animals are expected, based on our collaborators experience and published literature.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our preliminary pilot studies have allowed us to estimate the likely variation in the immune response and to choose a group size for future experiments. Further small-scale studies with new candidatevaccines will ensure that we can optimise the dosing regimes and the number of animals in each experimental group.

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 share tissues with other groups highly experienced in investigating the immune response to increase the data collected.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Protocol 1: We will inject mice under the skin with vaccine candidates to generate an immune response. The use of adjuvant and subsequent boosters may be required to enhance the immune response to the vaccine candidate, but we do not expect these to cause significant adverse welfare effects.

Protocol 2: GAS frequently colonises and causes infections in the human nasal passages, making this model in mice highly relevant to the natural disease process. It allows researchers to study the dynamics of infection and immune response in a site where GAS commonly resides. A nasal infection model enables the investigation of early-stage

immune responses and the development of vaccines aimed at preventing GAS colonisation before it progresses to more severe disease.

Protocol 3: A soft tissue infection model plays a pivotal role in advancing vaccine development efforts against GAS. It provides a controlled and relevant environment to assess vaccine responses, ultimately contributing to the development of effective vaccines for preventing GAS-related soft tissue infections, which can have a significant impact on public health. Mice will be injected with GAS into the thigh muscle.

We require information from both the respiratory infection model and the soft tissue model to target upper respiratory infections and improve our understanding of mucosal immune responses, as well as assessing protection against invasive diseases.

Why can’t you use animals that are less sentient?

We require adult mice in which the immune system is fully mature and to keep these animals for a few months in order to measure the development of the immune response to a vaccine candidate.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For all three protocols, we will use a clinical scoring system in order to monitor the mice during the study phases. Mice will be handled in advance of starting experiments, in order to acclimatise them. Procedures will be carried out by experienced and competent staff. Protocol 1 establishes the most suitable vaccine candidates and dose to use. This minimises the welfare cost and harm for the animals once they are challenged with GAS (Protocol 2 and 3, respiratory and soft tissue infection studies).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the NC3Rs for best handling and procedure practise, and will adhere to LASA guidelines for dosing.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project

I receive the NC3Rs email updates on recent advances, as well as staying informed via our local NIO and animal users group forum.

64. Control of matrix homeostasis in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

collagen, immune cells, fibroblasts, fibrosis, therapy

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description 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 investigate the molecular and cellular mechanisms of collagen homeostasis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Collagen accounts for approximately 25% of protein mass in the body, and is the most abundant protein that is deposited outside the cell (i.e. extracellular matrix), with a primary function of providing a physical scaffold. Abnormal production or removal leads to diseases like fibrosis (overabundance of collagen fibrils; accounts for 45% of all deaths in the developed world) or poor wound healing (insufficient collagen production). Thus understanding how cells control this normal balance is crucial to finding new efficient therapies.

What outputs do you think you will see at the end of this project?

Collagen is the most abundant protein in the human body, providing structure to organs with very different functions (e.g. tendons vs lungs). It forms what is called the extracellular matrix, which is a proteinous scaffold outside the cell providing support as well as influencing cell behaviour. Collagen deposition is a tightly controlled process; dysregulation underpins many pathologies and age-related conditions, including fibrosis and heart disease. Despite collagen's fundamental importance, therapeutics for diseases associated with collagen have been lacking, due to conceptual hurdles in understanding how collagen is assembled/removed.

Closing these knowledge gaps will form the majority of outputs at the end of this project.

The major output from this project will be the knowledge of how collagen deposition is controlled at the molecular level, by coordinated actions of different types of cells (fibroblasts, immune cells) that play major roles in health and disease. This research will further our understanding of fibrotic responses, and identify new targets for treating lung fibrosis. It will also deepen understanding of other conditions heavily involving the immune system and matrix, e.g. wound healing and cancer metastasis.

Additionally, it may also provide understanding on how to treat collagen-associated rare diseases (e.g. osteogenesis imperfecta, Ehlers-Danlos Syndrome), which usually manifests as genetic musculoskeletal defects. The findings of this work will be disseminated in peer-reviewed publications, to scientific audiences at conference meetings, and to the lay public via forums such as Twitter and presentations/conversations at public or patient engagement events.

Who or what will benefit from these outputs, and how?

My research is of particular interest to the academic community: it will tackle fundamental yet understudied questions in basic biology, towards an improved understanding, in an unbiased manner, of the protein networks involved in controlling how collagen are processed in fibroblasts and immune cells (e.g. monocytes, macrophages). This knowledge can then be applied to understand the disease- progression mechanism of collagen pathologies. It will also define the roles of monocytes and macrophages in collagen deposition. As such, several academic disciplines will benefit from this research in the 4-5 year time mark:

1) Researchers interested in matrix biology functions. There is a distinction between the secretion of collagen and the assembly of collagen into a scaffold, and they are separately controlled by the cells. The molecular insights that will be answered in this research will change the way matrix biologists associate collagen production to collagen functionality.

2) Clinical scientists working general fibrosis research. This work will greatly advance molecular knowledge on how a collagen scaffold is controlled, opening new avenues to treatment.

3) Immunologists studying how the matrix influence immune cells response and vice-versa. This research will identify the roles of monocytes and macrophages in collagen trafficking.

4) Academic clinicians who work on relevant diseases that are known to be exacerbated by collagen dysregulation, such as cancer, osteoarthritis, and cardiovascular diseases.

As molecular mechanisms are elucidated, new targets for therapies to treat collagen pathologies will be revealed, thus in the mid- to long-term (5-15 years) these targets will be able to be further tested with the aim to develop them for clinical application that will benefit patients.

How will you look to maximise the outputs of this work?

All data accrued in the project will be disseminated regardless of the outcome. We will publish the research output, as well as scientific approaches, in a timely manner in high impact open access journals to ensure the research will have the greatest impact. Where appropriate, the manuscript will be deposited on preprint servers (e.g. bioRXiv) to maximise availability to everyone. We will also attend and present research findings regularly, at scientific conferences to share and discuss results with our national and international colleagues, and establish new collaboration to maximise research impact.

Effective communication with established collaborators and any new potential collaborators will be ensured, in the means of online joint-lab meetings and delivery of seminars to a wide audience beyond this institution. We will also promote the research findings and provide lay summaries on Twitter, and present at internal and external seminars, and engage with the public about the research (e.g. outreach activities). We will also engage with with small focus groups who may benefit from our ongoing research (e.g. patients groups).

Species and numbers of animals expected to be used

- Mice: 2000 over 5 years.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In this project I will breed two mouse models. These are a well-established Per2::luc mice that have an internal reporter for circadian studies, and Nluc::col1a2 mice that have collagen-I tagged at a specific region (N-propeptide) endogenously.

Although most animals exhibit circadian rhythms, many of the tools and approaches for circadian studies were developed in mice. The circadian clock mechanism is highly conserved between mice and humans, and there is also much similarity between the physiology of the two species, thus mice are a good model organism to define processes that could then be inferred in humans.

As the Nluc::col1a2 mice are a new mouse strain, we will be characterising the strain at all ages to understand how the N-propeptide controls collagen homeostasis. For all other experiments, we will be using 12 week-old or older mice, to ensure that effects observed are comparable to that of an adult human patient.

Typically, what will be done to an animal used in your project?

The mice will be bred and maintained using standard protocols. All mice to be used for data gathering will be humanely killed. Mice will also be genotyped, which will most likely be done by ear biopsy, or by sampling either the blood or hair, or by mouth swabbing.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the mice will not experience any adverse effects. However one of our two strains will experience a moderate developmental phenotype which includes a domed head and short stature. However, with floor feeding, no additional adverse effects have been observed in our previous work. Some mice will experience an approximate 35% reduction of body size and weight, as a result of failure to grow to a full adult size (compared to wild type littermates). All mice will be closely monitored, continuously assessed for their body conditioning score (which gives a quantitative measure of body fat and muscle), and any general adverse effects beyond what is expected will lead to them 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)?

No severities for the Per2::luc strain.

Moderate severities for the Nluc::col1a2 strain.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project aims to study the extracellular matrix in health and disease, with a specific focus on collagen-I. Non-animal models do not form an extracellular matrix that is present in animal models and so animals are required for this specific project.

Which non-animal alternatives did you consider for use in this project?

1. in vitro 2D co-culture system.

2. in vitro spheroid cultures, which are 3D cell cultures.

Why were they not suitable?

All in vitro co-culture/spheroid culture systems only allows for 2-3 types of cells to be studied in terms of their interaction with one another, and lacks the complexity seen in animal models. Lack of blood flow to the cultures means that immune cell infiltration cannot be mimicked, and thus the dynamic nature of extracellular matrix regulation is lost in these non-animal systems.

Additionally, the use of animal models allow the discovery of new interactions central to matrix homeostasis. A great example is the creation of the Nluc::col1a2 mouse strain, where the in vitro cell system showed no defects on collagen deposition, but nevertheless the mouse has musculoskeletal defects, highlighting the dynamical nature of collagen homeostasis control that impossible to replicate in an in vitro system.

Once observations and subsequent hypotheses have been developed from the mouse studies, different types of cell culture systems will be used to establish the details of the molecular mechanism that drives these observations; these molecular mechanisms will then be verified in the mouse again to ensure the findings are correct even in complex organisms.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For primary tissues and cells, and from prior studies, we estimate we will require around 100 Per2::luc mice per year to allow for circadian time course studies. Each mouse yields 40,000 alveolar macrophages which will only be sufficient for 1 or 2 in vitro/ex vivo experimentation.

To get statistically significant results in the Nluc::col1a2 mice we must perform a cross between heterozygous mice to wildtype mice to study the heterozygous phenotype genotypes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The research team consulted with statisticians to advise on our experimental design.

Both genders of mice will be used, although recorded separately (in case there is any sex related variability in phenotypes), to ensure we are not biased towards any sexes in our reporting.

Primary measurements in this case is body weight, As we do not yet have data on variability of body weight of the het/homo lines at maturity, we utilised the best available data) to estimate the number we may require.

We will revisit power calculation again once more preliminary data has been collected, and reassess the sample size required. The number provided here is conservative and ignores

litter to litter variability, so as to ensure we have sufficient data to draw meaningful conclusions.

For both strains, we have established a breeding plan that will yield the required numbers of mice with the smallest number of crossings.

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 reduce the numbers of mice used in the experiments. Additionally, any tissues unused will be shared with other groups that are interested in circadian rhythm, or utilising primary cells for downstream in vitro studies. We have a track record of sharing tissues with other groups to maximize usage of the 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.

Mice will be closely monitored from birth to minimise animal suffering.

Floor feeding provides enrichment and allows shorter mice to reach food easily. With floor feeding, heterozygous Nluc::col1a2 mice can live up to a year without additional adverse effects. There are no malocclusions or noticeable behavioural differences between Nluc::col1a2 heterozygous mice and their littermates. Heterozygous mice do not have additional suffering; thus it is likely this mouse strain will allow valuable insights into how these rare diseases occur, while having minimal impact on the their overall well-being. Once pups are yielded they will be closely monitored, genotyped, and measured.

Any mice with additional adverse effects will be humanely killed to minimise pain, suffering, distress, and harm associated with that particular genotype.

Why can’t you use animals that are less sentient?

One of the key caveats of animal research is the use of immature animals which leads to incompatibility between what is observed in an animal to what is observed in humans, thus we are deliberating selecting mice of a more mature age to better mimic adult human (patho)physiology. Terminally anaesthetised mice will not allow for dynamic investigation of collagen homeostasis, and less sentient species (e.g. zebrafish) do not assemble collagen-I in the same way as mammals in their skeletons, which is the major matrix effector molecule that causes pathological progression.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Per2::luc mice are already a well-established mouse strain within the facility, and no additional procedures will be performed on these mice without a humane end point.

Nluc::col1a2 mice - post-genotyping, all mice will be monitored closely for adverse effects such as musculoskeletal development defects, dramatic loss of weight, and loss of appetite. We expect the heterozygous strain to have defects such as hydrocephaly and short stature, however with soft food the mice will not have any other adverse effects. Homozygous breeding programme will be closely monitored to ensure animals do not suffer due to the additional Nluc::col1a2 allele. This will be a general monitoring to ensure we are capturing any issues that we are not anticipating.

Animals in this project will have an humane end-point of around 12-14 weeks, with some that may extend to older age (e.g. 6-15 months) as long as there are no adverse effects on the health of the mice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

My ethos is to reduce waste, promote alternative, and increase reproducibility of my animal research, which is why I will continuously consult the NC3Rs website (https://nc3rs.org.uk/3rs-resources) for up- to-date best practice guidance, in particular with breeding and maintenance plans. Another website I plan to consult is the PREPARE guidelines (https://norecopa.no/prepare).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Continuous communication and consultation with the staff in the animal facility and with the NC3Rs through access to newsletters will ensure advances in 3Rs are captured. All lab members will be required to use the online resources available (with special emphasis on breeding and colony management) at the NC3Rs website (https://nc3rs.org.uk/breeding-and-colony-management) before beginning experimentation, and I expect all lab members to attend 3Rs workshops organised by the animal facility.

65. Regulation of axonal 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
- 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

Neuromuscular diseases, Neurodegeneration, Therapy, Axonal transport, Drug target

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aims of this project are to: i) investigate the mechanisms determining the onset and progression of debilitating and often fatal neurodegenerative diseases affecting the neuromuscular system, such as motor neuron diseases and inherited peripheral neuropathies, as well as forms of dementia, such as frontotemporal dementia and tauopathies; ii) identify novel therapeutic targets for these neurodegenerative diseases; and iii) develop novel therapeutic strategies to ameliorate the symptoms of these conditions and stop their progression. This is an urgent societal need since there is currently no cure or disease-modifying therapy for these neurodegenerative 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 will investigate pathologies of the neuromuscular and central nervous systems that affect motor neurons, nerves and muscles. These diseases comprise motor neuron diseases, such as amyotrophic lateral sclerosis (ALS), genetic peripheral neuropathies, such as Charcot-Marie-Tooth (CMT) disease, as well as central nervous system dysfunctions, known as tauopathies, which are due to the accumulation of a pathological form of the tau protein. Whilst familial motor neuron disease (MND) is due to genetic causes and is relatively rare, the majority of MND lacks a clear genetic component and is defined as sporadic. Nevertheless, the vast majority of familial and sporadic forms of MND share common pathological traits, such as the accumulation of pathological aggregates. MND is now recognised to be part of a disease spectrum affecting motor neurons alone, or in combination with muscle, or other neuronal populations in the brain, causing specific forms of dementia, called Frontotemporal Dementia (FTD).

The economic and societal burden of these neurological diseases is enormous. ALS, the most common adult-onset motor neuron disease, is a fatal, rapidly progressing disease with 30% of patients dying within 12 months of diagnosis and more than 50% within 2 years. On average, about 3,800 new ALS cases are diagnosed in the UK every year, with a total annual yearly cost in excess of £260M. Some ALS patients experience also the symptoms of FTD, a particularly debilitating form of dementia developing in midlife or earlier. Population studies show poor life expectancy of FTD patients with survival comparable to that of Alzheimer's disease, and a massive impact on family and carers. On average, more than 12,000 new FTD cases are diagnosed in the UK every year, with a total annual yearly cost in excess of £1.2B. The main symptoms of CMT, such muscle weakness, abnormal gait, numbness of the feet, instead start to appear in childhood or early adolescence. CMT is progressive and its symptoms become increasingly worse with age, making everyday tasks increasingly difficult.

On average, about 10,000 new CMT cases are diagnosed in the UK every year, with a total annual yearly cost in excess of £190M.

In spite of significant improvements in our understanding of the molecular mechanisms driving of these neurodegenerative conditions, very few therapeutic options are currently available. Sadly, these interventions show very limited efficacy in altering the progression and/or the outcome of any of these diseases.

This makes the development of effective therapies for MND and FTD a societal priority. Failure of several recent trials in MND has underlined the need for step changes in our approach to these conditions and in our understanding of the pathological mechanisms at play. Only by doing so, will we be able to identify innovative drug targets and therapeutic approaches addressing these disorders, bringing significant improvement to patient quality of life and clinical outcome.

What outputs do you think you will see at the end of this project?

The planned outputs of this work include:

i) Novel knowledge in the field of neuromuscular and neurodegenerative diseases, including ALS/MND, FTD/tauopathies, and peripheral neuropathies, such as CMT.

ii) Identification of novel drug targets and development of candidate compounds directed against these disease targets with the potential to be taken to clinical trials.

iii) Development of innovative therapeutic strategies, such as genetic therapies, for neurodegenerative diseases.

- iv) Publications of these results in scientific journals and where appropriate, filing patents.

- v) Presentations to scientific audiences, patient associations, and the public.

- Who or what will benefit from these outputs, and how?

The outputs of this project will benefit several diverse groups, including:

i) The components of our research group and affiliated group, in particular early career researchers, graduate and undergraduate students.

ii) Other researchers working in the field of neuromuscular and neurodegenerative diseases, including ALS/MND, FTD/tauopathies, and peripheral neuropathies, such as CMT.

iii) Clinicians working in the field of neuromuscular and neurodegenerative diseases.

iv) Patients affected by neuromuscular diseases and their families/careers.

v) The pharmaceutical industry and biotech.

How will you look to maximise the outputs of this work?

The outputs of the work described in this proposal will be maximised using different approaches, including:

i) By collaborating with academic colleagues, locally, nationally (e.g., via the national consortium United2End MND and the UK MND Research Institute, of which our group is a founding member), and internationally.

ii) By making data (e.g., whole unprocessed datasets and original data deposited in public repositories) and resources (e.g., cell and animal tissue) available to other researchers and requesting parties.

iii) By widely disseminating our discoveries, including publication in open-access journals, presentations at scientific meetings, patient information days, and public engagement events.

iv) By collaborating with the pharmaceutical industry and biotech.

Species and numbers of animals expected to be used

- Mice: 12,000

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.

In the proposed work plan, we will use mice, either wild-type or genetically modified to model as close as possible specific aspects of human neurodegenerative diseases. As most of these pathologies usually manifest in adulthood or late age, we will examine mice at various ages, including during development, since this will help us to pinpoint relevant early causes of disease. Furthermore, we will examine adult and aged mice, when disease phenotypes are likely to be established. These pathological symptoms will then inform us about the progression of the disease and how therapeutic interventions affect disease onset and progression.

Typically, what will be done to an animal used in your project?

Mice will experience mild, transient pain and no lasting harm from administering substances or genetic treatments, including novel potential therapeutic compounds, using standard routes (e.g., intramuscular, intravenous, subcutaneous, intraperitoneal, intraocular, gavage). Where administration is required for prolonged periods, when possible, animals will be treated orally by dissolving the substance into their drinking water or mixing it with their food. In cases where this is not possible, animals will be surgically equipped with slow-release devices, such as a mini-pump, a slow-release implant, or refillable iPRECIO pumps for extended applications.

Some mice will have minor surgery, for example, to implant a device under the skin releasing a potential therapeutic compound slowly over long period or an optical window or to induce nerve damage either in the spinal cord or peripheral nerve. These mice are likely to experience some discomfort after surgery and some mild to moderate pain, which will be pharmacologically managed with analgesics. Mice might experience mild and transient discomfort from the sampling of blood.

Terminal procedures (e.g., some forms of axonal transport studies) will be undertaken under non- recovery anaesthesia where mice will only experience mild distress and no pain.

To test the efficacy of potential new drugs, mice will be monitored at different time points by determining their body weight, and by assessing their neuromuscular function, for example, their ability to remain in equilibrium or their grip strength, using established devices. These measurements are not causing overt long-term adverse effects or distress, and as a result, animals will be returned to their cages at the end of these tests.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected impact of the procedures in this project is mild or moderate. Based on our experience with the approaches described in this proposal, we anticipate very few adverse effects. As stated, some mice will have minor surgery, for example, to implant an optical window, or a device under the skin releasing a potential therapeutic compound slowly over long periods. Animals are expected to recover quickly and experience no pain or long-

lasting harm since they will be treated, when appropriate, with painkillers and post-operative care until full recovery.

Some genetically-modified mice will express mutations causing neurodegenerative or neuromuscular diseases in human patients. These might yield functional deficits, such as mild behavioural alterations and muscle weakness. Periodic observation of these animals, including cage video monitoring, will be deployed to ensure their well-being and the absence of harmful phenotypes (e.g., seizures). For mouse strains modelling motor neuron diseases and peripheral neuropathies, functional deficits are usually restricted to hindlimbs and may include gait abnormalities due to muscle weakness. In such cases, mice will be provided with easy access to food and water, for example by providing a soggy diet within the home cage. In case the disease results in weight loss, mice will be regularly weighed once a week; if a decline in body weight is detected, they will be weighed daily to ensure that weight loss does not exceed 15%, in which case they will be culled.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of procedures in this project will be mild (75%) or moderate (25%).

For breeding and maintenance of genetically-modified mice, 50% of the mice will be indistinguishable from wild-type (sub-threshold), 25% will be mild, and 25% moderate.

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?

We would not be able to successfully deliver the aims of this project without using animals in our research. Data published in the last two decades clearly demonstrated that the pathogenesis of neurodegenerative diseases dealt within this proposal is not restricted to nerve cells, but also involves several types of non-neuronal cells within the brain and spinal cord, as well as muscle cells. To date, it is not possible to reliably model the highly complex interactions occurring between these multiple cell types in culture or using artificial intelligence/in silico modelling. Although the progress in in vitro culture of mammalian cells, including human induced pluripotent stem cells (iPSCs), has been very impressive, robust paradigms of ageing, a consolidated risk factor in neurodegenerative diseases, are not currently available for cells in culture as well as systems to study the impact of the immune system on cell survival.

Notwithstanding these considerations, we will make use of cell culture models to address the key aims of this project. Indeed, a major focus of our work during the course of this project will be the implementation of human cellular models of disease on a chip, thereby reducing the need for animal experiments.

Which non-animal alternatives did you consider for use in this project?

We have explored alternatives to replace animals in our experiments, such as the use of cell lines, and neurons derived from mouse embryonic stem (ES) cells and human iPSCs either derived from patients, or in which pathological mutations have been introduced in healthy iPSC cells to limit the impact of genetic variability in our pathomechanistic studies. Mouse ES- and human iPSC-derived nerve cells effectively replace nerve cells obtained from mice in some analyses, such as drug screens and preliminary tests to assess the consequences of removing genes on axonal transport and nerve survival. However, mice are irreplaceable for testing the effects of these mutations on the development of the nervous system, its maintenance in adulthood and to test the effects of novel drugs repairing axonal transport during the progression of neurodegenerative diseases.

Why were they not suitable?

In addition to in vitro models, several non-protected species, including sea squirts, nematodes, and fruit flies have been used to generate transgenic models of neurodegenerative diseases. Undoubtfully, these models have advantages, such as their suitability to carry out large-scale in vivo genetic screens. However, the mouse remains the system of choice to understand how impaired axonal transport contributes to nerve cell death and to evaluate the effects of novel drugs repairing these deficits in disease. This is due to the similarity in how mouse and human nerve cells work, and the vast range of mouse models closely mimicking human neurodegenerative diseases currently 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?

When assessing the total number of mice to be used in our programme of work, we use an established experimental design accounting for:

Group size. Group size will be determined based on published guidelines and previous data based on work performed by our team in the last two decades using similar protocols, models, and strategies.

For most of the planned experiments, consensus guidelines for how to conduct the investigation and the minimum number of animals required per experimental group have been established and published over several years by many investigators. For example, see 'Guidelines for preclinical animal research in ALS/MND: A consensus meeting' (Amyotroph Lateral Scler 2010 11:38-45). We will follow these guidelines as they are

accepted by researchers in the field and funders and are based on the best practice of over 20 years of animal experimentation in the field.

Group selection. This will include considerations on: i. gender, with the inclusion of male/female cohorts; ii. age-matched wild-type controls and disease-model groups; and iii. experimental (treated with different doses of the potential therapeutic compounds) and control (vehicle-treated).

Appropriate control groups. For experiments investigating pathological pathways and potential novel drug targets in animal models (Aim 1 and 2), the control groups will be wild-type littermates to control for the assessment of genetically-modified disease models. In the case of experiments involving surgical procedures (e.g., nerve injury), experimental and sham-operated mice will be included.

In the case of Aim 3 (Development of novel therapeutic strategies), four distinct groups will be used when testing the effects of novel potential therapeutic compounds: three controls (1. wild-type untreated; 2. wild-type treated; 3. model of disease-untreated) and one experimental cohort (4. model of disease-treated).

For functional testing of the neuromuscular junction, experimental groups will usually consist of n=10 animals per group/sex/time point examined. In some experiments, e.g., behavioural/lifespan/weight assessments, group sizes will be larger (up to n=20 animals per group/sex/time point examined). For some disease models, such as the mutant SOD1 model of ALS, the group should be sex-specific as disease progression is overtly modulated by gender.

Animals will be randomised into treatment groups and the experimenter blinded both at the time of treatment allocation and at the point of assessment, as routinely done in our team with the aid of a colleague not involved in the study.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will design our experiments according to the NC3Rs ARRIVE Guidelines and the PREPARE Guidelines (PREPARE: guidelines for planning animal research and testing. Lab Anim 2018 52:135- 141)

The experiments described in this project will be discussed with biostatisticians. In all aspects of the project, measures will be undertaken to minimise the number of animals used wherever possible without negatively impacting the statistical soundness of the data. For example, tissues from individual animals will be shared between group members and collaborators in the department and elsewhere, so that an individual mouse can be used to support experiments undertaken by several researchers, thereby reducing the total number of animals to a minimum.

In our experimental design, we have taken measures to account for the possibility of variability in our animal cohorts, and will minimise this variability by careful animal phenotyping. To cite a cogent example, longitudinal analyses of the mutant SOD1 model of ALS have revealed that pathology progresses faster in male than female mice. This unexpected finding stresses the need of assessing mice of both genders in preclinical testing of novel therapeutic compounds.

Careful phenotyping has also revealed that some behavioural assessments such as grip strength and rotarod testing have inherent variability and therefore larger cohorts (min n=12/group per sex) are needed to account for this variability and obtain reliable data. A few physiological and behavioural tests are also known to be experimenter-dependent, and so these tests will be carried out by the same researcher throughout the study to minimise variability.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We work closely and seek advice from the BSU staff to ensure the efficient breeding of all our colonies. Our senior lab manager and associated senior researcher have an excellent track record in managing mouse colonies and overseeing complex breeding programmes.

Wherever possible, we will reduce the number of animals used in this workplan by using stored tissue available in existing biobanks or by using surplus stock.

To optimise the number of animals in a specific study, we typically run pilot experiments aiming to test the underlying hypothesis using molecular and cellular models to identify pathological pathways/targets at play, and to establish target engagement. In experiments testing new therapeutic molecules, dose- response curves will be determined in vitro and ex vivo, so that appropriate concentrations will be used in vivo.

In this workplan, we will minimise animal numbers by maximising the data obtained from each animal, for example, by performing longitudinal tests or physiological analyses in animals in which axonal transport will be assessed before culling and removal of relevant tissues at the end of the experiment for molecular and histopathological analyses. We will harvest as many tissues as possible and if they are not required for immediate examination, we will freeze them and make them available to other members of the team, collaborators and colleagues working on similar questions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The work described in this project will be undertaken in mice, a mammalian system in which technology allows us to create mutations in any gene of interest and hence investigate the molecular, cellular, and tissue basis of disease pathogenesis. We also have a comprehensive understanding of the mouse nervous system and most of our previous work has been gathered from mice, which significantly reduces the number of animals to be used in this study. Mice expressing mutations that are known to be involved or cause neurodegenerative diseases studied in this project have already been created and new mice are in development.

Wherever possible we will use in vitro models, e.g., human iPSC models of neurodegenerative disease, which completely avoids the need for animal use. We also use models with milder disease phenotypes to test specific questions e.g., milder ALS strains with fewer transgene copies, which have a milder form of the disease than high copy number transgenic strains, or humanised model of disease in which the mutant human gene replaces the murine genetic locus, therefore avoiding overexpression of the mutant gene in a wild-type murine background.

Key aspects of pathology can be modelled without the use of genetically modified mice. For example, disruption to the neuromuscular system can be achieved by injury to peripheral nerves, such as the sciatic nerve, resulting in paralysis of the hindleg. Pharmacological agents can also be used to disrupt neuromuscular function. Furthermore, in the case of some forms of CMT, administration of the mutant human protein in wild-type mice replicate some of the disease phenotype observed in genetically- modified strains.

Overall, in this project, we will use the model with the mildest phenotype that replicates key aspects of disease pathology. For example, we will minimise harm during surgery by undertaking the mildest injury required to meet the scientific objectives and induce the required phenotype e.g., lesions will be unilateral, nerve crush injury will be used rather than axotomy where appropriate. In all cases, supportive therapy will be employed to minimise the impact of paresis or paralysis e.g., provision of a soggy diet in the home cage following surgery or when the disease phenotype reduces mobility, as observed in animal models of ALS (e.g., mutant SOD1).

A key deliverable of this workplan is the development of novel therapies to treat neuromuscular and neurodegenerative diseases. We will undertake pre-clinical testing of potential therapeutic agents, including pharmacological and genetic therapies. These will be administered to mice via the least

invasive route to minimise animal distress and will be determined by factors such as bioavailability, the site of pathology being targeted, and the stability of the therapy. Animals will be treated by mouth, through food, injection, or using implanted mini-pumps or drug reservoirs for long-term treatment.

As the human disorders we are studying typically manifest in adulthood or ageing, we need to study these disease models throughout the disease course, including up to humane endpoints, particularly to establish the effect of therapeutic approaches on when disease signs first appear (disease onset), as well as lifespan, as the human neurodegenerative diseases under study result in premature death.

Indeed, clinical trials rely on survival as an outcome measure of efficacy. We will carefully monitor the welfare of animals to ensure they are maintained in optimal physiological conditions, with regular assessment of their appearance, body weight, and disease signs such as muscle weakness. As the disease progresses, animals will be assessed daily. For diseases such as ALS, it is essential to use outcome measures such as disease onset, and lifespan, as these diseases are often sporadic and are not diagnosed until symptom onset. Therefore, any therapy that might prevent disease onset will not be of benefit to most ALS patients.

Why can’t you use animals that are less sentient?

Non-mammalian animals are limited in their use because they either do not have the same complexity of their neuromuscular or nervous system, and their immune system is substantially different from the human immune system to provide relevant results.

It is not possible to use embryos or very young animals in this project as the diseases we are studying manifest on an adult and often ageing background.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedures used in this project will be refined to minimise the welfare costs for the animals in the following manner:

i. We will closely monitor the animals for signs of disease onset. After disease onset has been detected, the monitoring will become more frequent, such as assessing the animal twice daily for adverse effects such as changes in weight, piloerection, paleness, grimace, and dermatitis.

ii. For animals that have undergone surgery, a post-operative care plan is put in place, which includes pain management and assessment of recovery.

iii. In specific cases, it may be possible to reduce the duration of the experiment by studying the onset of symptoms as an outcome, or disease severity at an earlier stage of the disease.

iv. As some of the experiments will include assessment of animals as they age, we will ensure that the group size in these experiments is sufficient to accommodate attrition - i.e., loss of animals-, and to avoid single housing determined by animal losses due to old age. Longer drinking spouts will be used, and animals will be monitored for adverse effects such as changes in weight, dermatitis, piloerection, paleness, changes in mobility, lumps, eye defects, abnormal respiration, or changes in the appearance and consistency of stools.

If these conditions are observed, animals will be treated accordingly, and if the adverse effects do not resolve, the animals will be culled.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have considered the PREPARE guidelines when planning our experiments, which build on the ARRIVE guidelines which we have previously followed (PREPARE: guidelines for planning animal research and testing. Lab Anim 2018 52:135-141). Furthermore, we will periodically access guidance and publications from the NC3Rs.

We have also based the design of our testing experiments on published guidelines for studies undertaken in mice that model ALS (Guidelines for preclinical animal research in ALS/MND: A consensus meeting. Amyotroph Lateral Scler 2010 11:38-45) which are accepted in the field by researchers, funders, and journals as the standard experimental design for preclinical research in MND.

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 are currently subscribed to the NC3Rs newsletter. In addition, we are signed up to receive regular updates from the organisation 'Understanding Animal Research' via the British Society of Cell Biology, of which I and other members of the groups are members.

Our Institution is fully committed to the principles of the 3Rs to minimise harm and discomfort to the animals and encourages and supports the implementation of advances in the 3Rs by Licence Holders. The Biological Services website has a dedicated 3Rs webpage, which notifies users of news and advances in 3Rs implementation.

66. The regulation of whole-body metabolism 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
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Obesity, Diabetes, Brain, Appetite, Body weight

Animal types	Life stages
Mice	adult, pregnant, aged, 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 the brain controls appetite and body weight. This includes normal regulation and what can go wrong with the development of diseases like obesity and diabetes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We are experiencing an epidemic in obesity and diabetes, including an alarming increase in the incidence of these diseases in children. By better understanding how metabolism is

normally controlled (that is how we control the flow of nutrients through our bodies), we can help to develop new treatments or improve those that are already available, but which may have unwanted side effects.

If an animal is metabolically challenged it will respond and adapt, bringing into play most body systems. We need to understand why and how metabolic adaptation occurs by studying it in different guises, including responses to voluntary interventions (such as dieting and exercise), pharmacological interventions (which may produce unwanted side effects), pathology (sickness and disease) or life events (such as pregnancy, torpor and ageing).

What outputs do you think you will see at the end of this project?

We will increase our basic knowledge of how the brain controls appetite and body weight. We will provide mechanisms of action of drugs currently undergoing development and which will assist in bringing them to market. We will provide novel targets for the development of new drugs. Our findings will be disseminated in scientific publications, at professional conferences and at meetings for patient groups.

Who or what will benefit from these outputs, and how?

The immediate beneficiaries will be the academic community who wish to understand body-weight regulation. Immediate benefits will also be to our industrial partners who will be able to use our discoveries to support their development programmes or, indeed, to bring programmes to an end. The latter is particularly important to reduce further experimentation and avoid the unnecessary use of animals. By separating different aspects of behaviour, the pathways involved and the factors that regulate them, we will also propose new interventions to bring benefit to those suffering with metabolic disease or who are struggling to control their body weight. We expect our findings to be adopted by partners in the pharmaceutical industry within the lifetime of the project. However, any potential commercialisation of target would require several years beyond the project.

How will you look to maximise the outputs of this work?

In addition to the dissemination of our findings outlined above, we will collaborate with pharmaceutical companies to understand how their drugs work and how we can overcome any unwanted side effects. On occasions, our findings may indicate that a drug is ineffective or unsafe. By maintaining our collaborations as academic, rather than commercial adventures, our findings will become accessible to the whole pharmaceutical industry and academia.

Species and numbers of animals expected to be used

Mice: 25000 mice bred; 9000 of which will be used in regulated procedures. The remainder will be used in non-regulated procedures and for tissue collection. In addition, some of the mice we breed will not have the necessary genotype for us to use in our experiments.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Although the brain's wiring is complex, it is very similar between humans and mice. This gives us the opportunity to use mice to understand normal and abnormal brain function. In fact, the breeding of genetically modified mice, in which we can introduce transgenes that control body weight, has massively accelerated our understanding and our ability to target metabolic pathways with drugs. A transgene is simply a modified piece of DNA that is introduced into specific cells and which then allows us to control their function, or perhaps look at the consequences of its introduction when the transgene reproduces a natural mutation found in humans. By using transgenic mice, we can employ minimally invasive techniques and we need far fewer experimental animals than in the past in order to progress knowledge. Our project intends to look at metabolism across the course of life and will include challenges which are normal (for example, pregnancy) or abnormal (for example, in response to disease or infection).

Typically, what will be done to an animal used in your project?

The transgenic mice we breed tend to grow and behave in the same ways as normal mice, though occasionally they may be a little fatter or thinner. To minimise any adverse effects, such as stress, we like to handle our mice (often daily) to get them used to being picked up. We may wish to alter their metabolic status, for example by restricting their food intake or, instead, by giving them a high-fat diet to eat. If we need to implant a catheter or a fibre into the skull or into a vein, this is done under general anaesthetic, after which the mice are allowed to fully recover. This means that, when the time comes for an experiment, we can give them an injection (either under the skin, into a vein or directly into the brain) without them hardly noticing. Usually, we will use a specific transgenic mouse which will allow us to change the activity of a pre-selected cell type in the brain. Thus, we might activate or silence those cells and look to see how the behaviour of the mouse changes. We can do a range of physiological tests on the mice, sometimes in their home cages, but often after acclimatising them to other cages.

Thus, we might put them in a scanner to see how much fat they have, measure their blood pressure by putting a tail-cuff around their tail (rather than their arm!), or measure their metabolic rate.

Occasionally, we even train our mice to poke their noses into holes to break an infrared beam or to press a little lever, which provides them with a sugar reward. This can tell us about their motivation to eat. Invariably, the parameters we measure are much simpler: for example, how much food do they eat or how much sugar is circulating in their bloodstream. For the latter, we can take pin-prick samples of blood from their tail and measure these in a sugar monitor, rather similar to how a diabetic patient would. We are very interested in why we lose our appetite when we are ill. This could be because we have an infection or because a drug has an undesirable side effect. Thus, on rare occasions, we will induce an illness, such as the flu or inflammation of the bowel. By understanding why these situations cause nausea and sickness, we hope to suggest treatments which will help improve symptoms.

What are the expected impacts and/or adverse effects for the animals during your project?

As stated above we strive to minimise any stress to our mice by getting them used to handling. We need to do surgery on about a fifth of our experimental mice in order to

manipulate how the brain responds to different signals or if we want to control the activity of pre-selected cells. In this case, we carry out the surgery with the mice under general anaesthetic, plus we give the mice pain killers and sometimes local anaesthetics, to make sure that they do not feel any pain. The mice recover very rapidly, so they can be returned to their home cages to carry on living as normal.

However, to study a disease it is often necessary to induce that disease artificially. This could be relatively benign, such as feeding mice a high-fat diet to make them put on weight. Mice adapt to being overweight and most of our animals will never develop any of the extreme complications associated with obesity, such as high blood pressure or diabetes. Conversely, many common diseases or the drugs used to treat them cause nausea and weight loss. Sometimes we will need to make manipulations to cause these adverse effects, so we can compare how the brain responds and how it is different to normal responses to eating a meal. Usually, the anorexia or nausea experienced by the mouse only lasts for a few hours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Our project requires the breeding and maintenance of several mouse lines. Every time a litter is born, about half of the pups will be “wild type” and the other half will be “transgenic” (i.e. they have small piece of their DNA that is different to the normal, wild-type mice). Usually, the transgenic mice will be perfectly normal and so are classed as not being in a severity bracket (sub-threshold). However, sometimes they may have a mild trait (e.g. slightly fatter than wild-type mice) or we may make a mild intervention to them (such as giving them an injection or putting them on a high-fat diet). We estimate that about 80% of the mice we breed for our project will remain within or below the mild bracket of severity. Very rarely, we will generate a transgenic mouse which may develop an unforeseen trait.

Assuming that this trait remains within the mild or moderate brackets, we may wish to justify maintaining this line in order to study it further. We estimate that about 20% of the mice we use in this project will undergo some form of recovery surgery, most commonly so that we can later manipulate cells in the brain. The mice will experience some weight loss and mild discomfort as they recover from the surgery, but this soon passes. The mice are normally kept for several weeks before we make any other manipulation, during which time we can acclimate them to handling or sometimes different cages. Occasionally, we will need to make a manipulation that we know will cause the mouse some discomfort. For example, when we are examining brain pathways causing anorexia, we may induce nausea or sickness-like behaviour. In these instances, and each occasion involving recovery surgery, the severity bracket will be classed as 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?

It is impossible to study appetite, body-weight regulation, circulating factors or responses to drugs in anything other than a normally behaving animal. When an animal is metabolically challenged it will respond and adapt. Normally, this will involve the brain interacting with other organs or systems.

Which non-animal alternatives did you consider for use in this project?

We would if we could, but there are no non-animal alternatives that would allow this project.

We can still find out a lot about brain cells by studying them isolated from the rest of the body. We have to kill the mice humanely, but this allows us to take slices of brain and put them in a dish. We can then record the minute electrical or chemical activity of individual brain cells. To enable us to identify the right cells in the complex brain, we have bred transgenic mice in which specific cell types glow fluorescently under our microscopes.

There have been major elements of discovery in our research ranging, for example, from the use of deep RNA Sequencing of transgenic neurons ex vivo to describe novel signalling molecules through to collaborating with clinicians to screen obese populations for previously unknown gene associations.

Why were they not suitable?

There are no alternatives, such as cell or organ cultures which will allow the study of physiology and behaviour. Unless a model shows normal physiology and behaviour, we cannot study regulation, pathology or treatment. Furthermore, an ultimate aim is that we can make a difference to the human population and, therefore, we need to utilise the animal model which provides the best, overall translation to humans. Basic mouse physiology and behaviour is very similar to those of humans, plus we are able to make use of our vast range of transgenic mouse models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers are based on our usage during five previous years. This is likely to be more accurate that trying to estimate numbers from as yet unplanned experiments in the following five years. The major determinant is the number of mice bred, not the number used in individual experiments. The level of breeding depends on maintaining over 60 different lines/crosses of mice and providing enough individuals to carry out all of our experiments. Several of these lines are used across multiple experiments by multiple

users. However, many of the lines are crosses and are bespoke to a particular experimenter. By using these bespoke lines, we are able to reduce the overall number of mice used in experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For an individual experiment, data provided from similar studies in the past or from pilot studies, allows us to make precise calculations of the minimum number of animals we will need to provide robust experimental results. When a line is not used regularly, we reduce the colony to a minimum or we end their breeding, having first cryopreserved sperm, eggs or embryos for future regeneration. By publishing our results in open access forums, we are obliged to share these resources with other laboratories, preventing the need to replicate line generation.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Whenever possible we use within subject comparisons (crossover designs) where an individual mouse receives both control and experimental treatments. This increases both the quality and statistical power of an experiment. Often, multiple parameters are measured in the same mice concurrently, which is possible due to our investment in complex behavioural and metabolic apparatus. This avoids the need for repeat experiments and also improves the value of collected data. To validate our mice post mortem (e.g. for the accuracy of surgical implants or injections), we administer a relevant stimulation before culling. Often this provides us with a further opportunity to collect additional data. We normally collect a number of tissues which provide data for the ongoing or additional experiments. On occasions, we can provide tissues from our experimental mice 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.

The majority of experiments will use transgenic mice which will allow us to manipulate pre-selected cell types in the brain very accurately. We will be able to stimulate or inhibit cells selectively, or record from them. We will measure a number of physiological and behavioural outputs. In the last five years, we have used over sixty different mouse lines/crosses. However, by using these approaches we have massively refined experiments, so that mice do not experience the same adverse effects as older models/approaches.

Why can’t you use animals that are less sentient?

It is impossible to study appetite, body-weight regulation, circulating factors or responses to drugs in anything other than a normally behaving animal. Using immature or anaesthetised animals would invalidate translation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

One major advance has been the use of remote radiotelemetry. This is where, during surgery, we implant a small radiotransmitter under the skin or in the abdomen of the mouse. Later, these devices allow us to monitor things like body temperature, blood pressure and brain activity without having to disturb the mice. Another example is to use the smallest needles possible when giving injections. In a more recent set of experiments, we have worked with our colleagues from a pharmaceutical company to refine further the use of “EpiPens” instead of normal needles – similar to children who suffer with allergic reactions. A notable recent improvement/Refinement is adoption of the Phenomaster system which maintains the mice in their normal, home cage, but allows the measurement of several behavioural and metabolic parameters at the same time (e.g. food and fluid intake, body weight, metabolic gases by indirect calorimetry and locomotor activity). This also means that fewer mice are required to gather the same information, leading to substantial Reduction in the number of mice required.

We now use transgenic mice to identify, control or record the activity of individual cell types in the brain. This allows us to determine how different cells respond to stimuli and how they communicate with each other without using the very invasive old techniques. Since we can manipulate the mice

while they are still in their home cage, we can record their behaviour, whether they are secreting hormones, or if their metabolism changes, with minimal disturbance. To do this we breed mice that have so-called “designer” proteins expressed in just a single cell type. The designer proteins lay dormant and the mice behave as usual. But, by then giving the mice a “designer” drug or by shining a light of precise wavelength through an optic fibre, we can activate or inhibit specific brain cells selectively, while studying changes in the mouse’s behaviour or physiology. All the time, our techniques are improving and our equipment is miniaturising, so it is now even possible to see and record the activity of individual brain cells in freely moving mice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We are informed by publications from NC3Rs, LASA and both the ARRIVE (https://arriveguidelines.org/) and PREPARE (Smith et al., 2018, Laboratory Animals 52: 135) guidelines for planning animal experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Members of the laboratory are fully integrated into the community of in vivo scientists both within our establishment, but also at other institutes around the world. Members of the group attend and present at 3Rs focussed seminars and workshops. In addition, local interaction with husbandry staff and named officers occurs on a daily basis. Staff within the animal facility have in the past and are always welcome to attend our lab meetings, where ideas are exchanged.

67. Breeding and Maintenance of GA Mice

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Breeding, Genetically Altered, Mice

Animal types	Life stages
Mice	adult, juvenile, neonate, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To breed and maintain genetically altered mice to be used on other project licences that don't have permission to breed, this will result in better management to ensure higher animal welfare and scientific quality.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

An increasing number of investigators require access to a service to efficiently and effectively breed, maintain, cryopreserve and reanimate GA rodents, at a specified pathogen status. This licence will enable the supply of whole animals or tissues to the biomedical research groups within the establishment, or occasionally to external researchers requiring lines held in our facility. The Breeding Core supplies wildtype and GA rodents to support research programmes, while our In vivo services team provides services for rederiving imported rodents, and archiving sperm and embryos from rodents. This PPL will ensure that the technical and welfare responsibilities of the two interrelated

services will be controlled centrally by the PPL holder, allowing for the development of an effective and efficient service whilst delivering the principles of the 3Rs (principally Reduction of the numbers of animals used).

What outputs do you think you will see at the end of this project?

GA rodents for use under separate authority or for future breeding stock.

Tissue from GA rodents for use by research colleagues who themselves may not hold licence authority under ASPA.

A biobank of cryopreserved rodent sperm and embryos.

Reanimation of cryopreserved sperm/embryos and rederivation of lines by embryo transfer.

Who or what will benefit from these outputs, and how?

The GA rodents and/or tissue produced will be used by academic staff members at the establishment. External collaborators and partnering institutions undertaking work at the establishment may also use the breeding service. The animals bred will be used in research projects currently requiring GA rodents produced under the existing project licence authority that this licence will replace. Such project licences cover a wide range of research programmes for example: - kidney disease, infection studies, cancer research.

How will you look to maximise the outputs of this work?

Encourage scientists to publish and attend workshops to share their findings including unsuccessful techniques. Introduce into the cryopreservation programme a process for researchers to share transgenic lines, for example after they have completed their programme of work we will have a process that automatically sends frozen material down to EMMA which is the national mouse archive. These store the material for two years before making the material open to others in the industry. We will include this process when sharing our methods of best practice at industry focused conferences.

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.

Much of the in vivo work at this research establishment requires the use of mice. Therefore, this breeding and supply licence, by definition will require the use of mice at all life stages.

Typically, what will be done to an animal used in your project?

Protocol 1 Superovulation: Females will be used around 6 weeks of age. They will receive two intra- peritoneal injections typically 48 hours apart. Animals may experience mild, transient pain and no lasting harm from the administration of substances by injection using standard routes (IP). They will either be mated or not depending on the requirement.

Protocol 2 Embryo recipient: Females will be mated to a sterile male, once a copulation plug is evident from the act of mating, she is now pseudo-pregnant. She will have a brief non surgical procedure under general anaesthesia using aseptic technique by someone experienced in the procedure for the implantation of embryos. This will cause the animal to experience some short-lived discomfort which will be treated will analgesics. Females maybe mated to a sterile male several times under this protocol but will only have the non surgical procedure on a maximum of 2 occurrences if unsuccessful on the 1st attempt. Before the the second attempt the NVS will be consulted.

Protocol 3 Sterile male: Males will have a scrotal vasectomy under general anaesthesia using aseptic techniques by someone experienced in the technique. These animals may experience some discomfort after surgery and some mild to moderate pain which will be treated with analgesics. When the sterile males aren't actively being used, they will be housed with a buddy. Typically a sterile male will live for 8 – 12 months. If they are too aggressive or don't carry out successful matings they will be removed from the colony and replaced.

Protocol 4 breeding:- Females will be used for breeding for a maximum of 6 months, males for 12 months. Offspring produced from these matings will either continue on this licence under other protocols, will be transferred to other project licences with authority to receive and use these strains or be maintained for tissue collection following schedule 1 to be used in ex-vivo work.

Typically, any animal that will undergo a surgical procedure will be trained to take fluids orally before surgery so they will take the analgesics orally after the procedure to reduce the stress after surgery where appropriate.

What are the expected impacts and/or adverse effects for the animals during your project?

Protocol 1: No adverse effects are expected.
Protocols 2 and 3: No adverse effect expected but due to the surgery element of the protocol, these animals may experience some discomfort after surgery and some mild to moderate pain which will be treated with analgesics. Any animal with unexpected adverse effects will be humanely killed.

Protocol 4: No adverse effects 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)?

Protocol 1 Superovulation:- Mild 100% of animals used in protocol. Protocol 2 Embryo recipient: Mild 100% of animals used in protocol. Protocol 3: Sterile males: Moderate 100% of animals used in the protocol. Protocol 4: Breeding and maintenance of GA mice (Mild) 10% mild and 90% sub- threshold

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are providing a service for researchers that don't have the skills or ability to carry out the process of breeding and maintaining a GA mouse colony or cryopreservation. Mice are biologically and physiologically well characterised genetically and can be genetically manipulated to mimic many human traits and diseases. As yet there are no alternatives to using whole animal models for certain areas of research.

Which non-animal alternatives did you consider for use in this project?

The purpose of this licence is to provide animals for other PPLs. The holder of the receiving PPL will be required to provide information on non-animal alternatives.

Why were they not suitable?

The purpose of this licence is to breed and supply mice for use in in vivo research which has been justified and authorised separately. Replacement is the responsibility of the holder of the receiving project licence

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers are estimated based on the current workload of the facility and animal usage under the previous service licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will work with the NC3R's and any other establishments that are already using the required techniques to ensure we are using the best practice.

Efficient breeding programmes.

By having a central colony of sterile males we will reduce the number of animals having surgery.

Ensuring our technicians are update with CDP regarding mouse breeding/ colony management.

Annual reviews regarding mice bred under this licence

Ensure that animals are moved onto receiving PPL in a timely manner.

Efficient management of the cryopreservation programme.

Tissue sharing.

Promote the use of the NC3R's experimental design assistant (EDA) to the receiving project licence holders.

Give advice to receiving project licence holders regarding blinding and randomisation of their studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Use younger females for the cryopreservation to maximise gametes retrieved

Efficient breeding programmes.

Tissue sharing

Attend relevant training courses to ensure the most relevant techniques are being used

Communicate via NC3R's

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 as these are the animals used at this establishment

Ensure good breeding programmes are in place and reviewed annually

Strains expressing mild phenotypes will be monitored by trained technicians to ensure early humane end points are used.

Review surgical care plans with NVS.

By using age appropriate females and good techniques we aim to maximise the gametes produced by superovulation.

Male mice that are genetically sterile are available and during the life of this project we plan to trial the use of these animals to reduce or possibly discontinue the need to create sterile males surgically

Why can’t you use animals that are less sentient?

Mice are required for use in other authorised studies at this establishment.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Typically, any animal that will undergo a surgical procedure will be trained to take fluids orally before surgery so they will take the analgesics orally after the procedure to reduce the stress after surgery where appropriate.

Routinely review the analgesics and post operative monitoring periods with the NVS for the surgical animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Using the Home Office document - Efficient breeding of Genetically Altered animals assessment framework when working out breeding plans.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By attending industry conferences and workshops. Keeping update with updates on websites and journals published by the relevant sources such as: ASRU, NC3R’s and Norecopa.

Project licence holder is an active member of the 3R’s focus group at the establishment.

68. Characterisation of Brain Tumours: from biology to druggable biomarkers

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, Brain tumour, Brain Metastases Diagnosis, Combination therapy

Animal types	Life stages
Mice	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify biomarkers, biological signals that can help us detect and target brain cancer as well as monitor whether a treatment is working, which can provide a foundation for developing new brain cancer therapies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Tumours of the Central Nervous System (CNS) are among the most feared cancers because they affect the very organ that defines personality and assures physical and cognitive independence. Once diagnosed, the current treatment options fail and most patients with malignant brain tumours succumb to their disease. For example, patients

diagnosed with glioblastoma (GBM), the most prevalent and lethal primary brain tumour, have median survival rates of only approximately 12-15 months despite maximal therapeutic intervention. The incidence of brain tumours continues to increase. It has been reported that that age-standardised incidence rates increased globally by 17·3% between 1990 and 2016. While therapeutic strategies have improved for several non-CNS tumours, current treatment for brain cancer patients offers only palliation.

The growing recognition of the brain representing a unique tumour microenvironment and the increasing inclusion of patients with brain tumours in clinical trials set the stage for a new decade of dynamic cancer research. A better understanding of the cellular and molecular features critical for the maintenance of brain tumours, the development and use of physiologically and clinically relevant pre- clincal models, should facilitate the identification of novel therapies and so enable personalized treatment strategies for patients suffering from this deadly disease.

What outputs do you think you will see at the end of this project?

There are several benefits expected with this project. The main benefits are that we are building platform of evidence and data critical for the development of treatments for brain cancer patients where most patients only receive supportive or palliative care. This project aims to aid the discovery and deliver the proof-of-concept preclinical data to permit clinical application of newly discovered therapies. During my time at my previous establishment (August 2020-2023) such research data supported the opening of a Phase I study. We have several studies focused on novel druggable biomarkers for which we have completed the initial proof-of-concept (studies were carried out by my team at an overseas research centre under the local Laws on Animal Experiments). More studies are needed which will be completed on this new licence application to get these potential therapies ready for the clinic. Key results from work carried out under this licence application will be published in the form of scientific research papers and/or presented at national/international meetings in accordance with ARRIVE guidelines to maximise the impact and ensure the reproducibility of our work.

Who or what will benefit from these outputs, and how?

The outputs of this study will benefit not just the scientific community (researchers, the pharmaceutical industry, clinicians), and potentially, in the longer term, patients.

How will you look to maximise the outputs of this work?

Our findings will be made available to other scientists through collaborations, publication in high-profile peer-reviewed journals and presentations at scientific conferences and meetings. Our Establishment has a policy of ensuring that all publications generated are available on open access to all. In addition, our work has direct translational and clinical applications that we will investigate through collaborations with clinicians at the Establishment and other medical cancer centres worldwide. Data will also be shared with the general public through outreach activities within the local community, social media and other public engagement activities.

Species and numbers of animals expected to be used

- Mice: 3300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice have been chosen for the study because they represent the least sentient species from which meaningful experimental data can be generated. Mice are widely used for in vivo drug development and correlative studies indicate the potential for the results to translate between the species. The human and mouse DNA content and organization are approximately similar, and display an equivalent number of genes, which share similar functions. Definitively, mouse models are important for placing the findings of in vitro (test tube) studies or comparative analysis of human samples into an appropriate and meaningful in vivo (living organism) context. It is the combination of in vitro, in silico (computer) and in vivo studies that provides the insight needed to understand cancer biology and to develop new therapeutic approaches. There are no effective approaches to hand that can replace the in vivo studies, as these allow the in vitro findings to be tested in an appropriate living organism. We study adult brain tumours and therefore we will use only adult mice. We will use both normal mice and mice lacking a complete immune system to allow use of human cancer cells.

Typically, what will be done to an animal used in your project?

Typical experiment (70% studies under this project licence)

The injection of tumour cells at a superficial site (for example subcutaneous injection/under the skin)

Tumour growth will be monitored regularly.

Therapy administration (for example abdominal injection) over a designated timeframe monitoring tolerance via weight and measures of well-being.

The mice will also have blood or tear samples in accordance with good practice.

The experiment will be finished when the tumours reach a size (~1.20 cm^3 or below).

Comprehensive experiment (30% studies under this project licence)

Anaesthetise mouse and inject tumour cells at a non-superficial site requiring surgical procedure (eg intracranial implantation into the brain).
Monitor tumour growth using non-invasive imaging (for example bioluminescence in vivo imaging).

Therapy administration (for example radiotherapy alone or in combination with targeted therapy) over a designated timeframe monitoring tolerance via weight and measures of well-being.

The mice will also have blood and/or tear samples taken.

The experiment will be finished based on an imaging-defined endpoint prior to any detrimental neurological signs being observed.

Prior to killing the mouse, administer pathophysiological detection-markers to aid determination of the impact of intervention on targeted tumour biology.

In all studies, mice will be randomised into groups and housed in ventilated cages which have their environment enhanced with items such as tunnels, houses, nesting material and gnawing blocks. At the end of any protocol, mice will be killed humanely.

Some studies will be undertaken in older mice (12 months of age) in order to reflect the age profile of the population that experience brain tumours. Brain tumours can start at any age. The risk of developing most cancers, including brain tumours, increases with age. The risk of brain tumours is greatest in those aged between 85 and 89 years.

What are the expected impacts and/or adverse effects for the animals during your project?

In the case of non-superficial models (Protocol 4), mice will undergo surgery to implant cells for example into the brain. They are expected to recover quickly and will be given painkillers and post- operative care just like people recovering in a hospital. Under these circumstances we regularly monitor animal progression very carefully and manage their treatment to minimise the potential pain or discomfort, taking advice from veterinarians and highly experienced animal technicians. The growth of tumours will be assessed by non-invasive in vivo imaging (using bioluminescence measurement).

In the case of superficial models such as the generation of subcutaneous or intradermal tumours (Protocol 3), tumour growth will be monitored regularly using callipers and once volume reaches 2/3rds of the licence limit the frequency of measurements will increase.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Based on our experience, the anticipated severity proportions for this project are:
90% moderate (mice will be implanted with tumours, growth monitored; and undergo surgical procedures with recover anaesthesia followed by drug treatment and/or radiation therapy accompanied by the collection of blood and tears). 10% mild (tumour-bearing mice receiving vehicle injection only).

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 develop improved therapies for brain tumours. We have analysed brain tumour- specific proteins using human tissue samples collected at surgery and based on this data, using cell culture models, we have identified possible new strategies to target brain cancer cells and so prevent brain tumours from growing. Initially, we plan in vitro (studies performed using cells cultured in a lab) studies to perform so-called target validation (here, we test the response of brain cancer cells to a specific therapy in a Petri dish) and to evaluate the effects (changes in survival, growth, ability to spread and invade) on relevant pre-clinical models such as primary cell cultures. Whether or not this will lead to the ultimate aim of reducing the growth and spread of a tumour harbouring the defect can only be effectively determined in an animal model.

The mouse is a model organism that closely resembles humans. The human and mouse genomes are approximately the same size, and display an equivalent number of functionally conserved genes.

Definitively, mouse models are important for placing the findings of in vitro studies or human tissue biopsy analyses into an appropriate and meaningful in vivo (studies performed in a living organism) context. This would be for example i) invasion of brain cancer cells into the distant part of the brain (such invasive behaviour is known to prevent complete surgical removal of these tumours in humans); the state of blood-brain barrier and its impact on drug exposure and so tumour growth inhibition; iii) the interplay of stroma/tumour microenvironment with cancer cells and how it impacts response to therapy.

It is the combination of in vitro and in vivo (living organism) studies that provide the insight needed to understand cancer biology and develop new therapeutic approaches, and there are no effective approaches to hand that can replace the in vivo studies, as these allow the in vitro findings to be tested in an appropriate environment.

Which non-animal alternatives did you consider for use in this project?

Thorough literature search will be performed prior any animal experiments to avoid data duplication/repetition of published studies. This will be done by browsing public repositories such as PubMed or Google Scholar as well as discussing with collaborative network and by attending seminars/conferences where possible. Next, the generation of pre-clinical in vitro (grown in the laboratory in a dish) models will be employed and used as an alternative approach to assessing responses for putative targets identified in our studies. The most promising targets pre-validated in vitro (n=1-3) will be then assessed in mice (in vivo).

Why were they not suitable?

The study of cells in culture (in vitro) and less sensitive organisms provide us with clues on the mechanisms of cellular processes in a simple and valuable context, which allows the establishment of hypotheses regarding the function of cells in a living animal. Whilst enormous progress has been made in the field of cancer research using in vitro models,

there are a number of questions that can only be addressed using animal models of disease:

drug delivery – can the compound get into the tumour?

modification – is the drug inactivated or activated by some process of metabolism?

toxicity – are there side effects that cannot be determined in vitro?

Microenvironment- are there other factors in the tumour environment that determine activity (e.g. other cell types that play a role). The complexity, dynamics, and whole organism level regulation of the immune response to cancer cannot currently be effectively modelled outside of a living organism.

Thus, to investigate the relationship between the immune system, cancer progression and response to anti-cancer therapy, we need to perform studies in living organisms with an immune system that functions similarly to that of humans. The immune systems of lower-order organisms such as nematode worms and fruit flies are too divergent from humans and frequently lack key elements such as adaptive immune cells that are critical for anti-cancer responses in humans.

Explant models have currently not developed to the point where they are comparable to animal studies for immune therapy, although this field is rapidly advancing, and we will follow this progression and consider these as alternative approaches where possible.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Before carrying out any studies, we will review historical in vivo data generated in our laboratory (and key learnings from these) as well as published data in the literature, as we have done when preparing this project licence application, we will continue consulting biostatisticians to ensure that all studies are appropriately powered. When investigating novel therapeutics with limited safety and/or efficacy data, we would start with tolerability and pharmacokinetic studies prior to executing a fully powered experiment. Based on a two-sample t-test, it was estimated that a minimum of 7 mice per group will be required to detect a desired 30% difference in tumour size, assuming a standard deviation of 20%, a significance level of 0.05 and a statistical power of 80%. Taking one extra mouse as a contingency, we will include 8 mice per group in this study.

Because brain tumours are known to be composed of multiple subpopulations with different drug sensitivity (each patient may have a different composition of such subpopulations), we would typically examine more than one model (at minimum two) to

ensure data robustness and translatability of our findings. Likewise, we may use several doses of a drug, or several different drugs or treatment combinations to test a hypothesis.

Rigorous experimental design considerations will be employed in the conduct of all experiments. In general, bias avoidance approaches will be used e.g. random assignment of animals to different treatment groups in a way that balances any important pre-treatment metrics and potential sources of bias. Where appropriate treatment assignment will be blinded to the data analyst (efficacy studies).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The use of mice will be minimised in several ways:

By considering ongoing statistical estimation of power requirements in each of the studies, using prior results in order to use the minimum number of mice while retaining sufficient numbers for statistical significance. In general, we will use a sample size capable of detecting a 30% practical difference with 80% power and 95% confidence.

By incorporating as many test groups as possible within a single controlled experiment, reducing the number of controls required compared to a series of smaller experiments.

By utilising tissues from different sites on one mouse for both treatment and control samples.

With internal tumours we will use non-invasive imaging where possible to assess parameters such as tumour volume this allows us to assess internal tumour volume without the need to humanely kill the mouse. This allows us to use the minimum number of animals to provide statistically appropriate studies.

Experiments will be designed using the principles outlined in the experimental design tool on https://eda.nc3rs.org.uk/ (such as randomisation, power, and blinding). PREPARE guidelines (https://norecopa.no/prepare) and reported following the ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

By doing as much preliminary work as possible in culture, 3-D spheroid and primary cell line models in vitro and in silico (computer-based) analysis before engaging in in vivo studies.

By minimising variability in results through utilising appropriate mouse strains inbred strainsand by housing them under identical conditions to limit variability.

By performing pilot studies using small numbers of mice when information is lacking in the literature/from collaborators so that the number of mice utilised in experiments is reduced to minimal levels.

By running experiments in parallel so that they can share a single control arm where possible

By taking care to ensure that each experiment is appropriately analysed and that the maximum amount of information is gathered, thus reducing the need for experiments to be repeated.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

70% of studies under this project licence will involve the injection of tumour cells or tumour fragments under the skin. Mice will be monitored for tumour growth and killed humanely when the tumours reach a size ~1.20 cm3. Based on experience at the Establishment, this is a size that typically does not cause pain or suffering.

Therapy administration will be given (for example abdominal injection) over a designated timeframe with a minimal dosing frequency that provides full therapeutic cover. Tolerance will be monitored via weight and measures of well-being of the mice.

30% of studies under this project licence will involve studies where tumour cells will be administered in the brain by intra-cranial implantation under recovery anaesthesia and given analgesia.

Tumour growth in the brain will be assessed using non-invasive (bioluminescence) imaging under brief recovery anaesthesia, to allow serial measurement in the same mouse. The mice will also have blood (using micro-sampling) or tear samples taken in accordance with good practice. Collection of tears requires only mild restraint and requires no needle. This approach to brain tumour research (orthotopic intracranial, intrathecal, intracardiac or intrathoracic injection of cells) is essential for the assessment of therapeutic responses in the context of tumour microenvironment. Tumours grown under the skin do not have the same tumour niche composition as brain where brain tumours grow naturally. Therefore, this variable would be missing without the use of the intracranial (brain) models. To minimise numbers of mice used, we will employ brain imaging under brief recovery anaesthesia to closely monitor tumour growth.

These models cause the least pain, suffering, distress or lasting harm given that we need to cause tumour growth, either under the skin on the flank or within the brain. As much as possible will be done using skin flank models of tumour growth.

We will continue to look for refinements in experimental protocols wherever possible, to minimize the potential suffering of mice used. Throughout experiments, we will monitor tissue and tumour growth and the health status of transplanted mice daily. Any mice that have health-related issues related to the tumour, surgery, transplantation or experimental therapy will be given immediate attention to alleviate symptoms or discomfort and will be

killed humanely (Schedule 1) if adverse effects are unexpectedly worse than anticipated or approaching the severity limit for that Protocol.

Why can't you use animals that are less sentient?

The mouse models (both immunocompetent and immunocompromised) allow us to faithfully recapitulate human disease and thus to understand the molecular and cellular events and steps involved in the initiation and progression of cancer as well as responses to anti-cancer therapy. The mouse is the least sentient animal which has sufficient similarity to humans for the purpose of our study. This is critical for increasing our understanding of cancer biology and developing therapies that can be translated to the clinic.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We constantly work to improve husbandry and procedures to minimise actual or potential pain, suffering, distress, or lasting harm and/or improve animal welfare. Animals will be groups housed wherever possible, provided with enrichment, and handled by appropriate techniques. Animal suffering will be minimised by making every effort to keep the tumour models employed at the subclinical levels. Wherever possible, this will be achieved by using non-invasive imaging modalities to monitor internal tumour growth and the development of metastatic disease. In addition, as detailed in the individual protocols, steps will be taken to minimise the severity of the procedures. Finally, we will ensure that all mice receive the highest standard of care, and preventative medicine (including anaesthesia and analgesia where required) will be used.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Surgical procedures will be carried out according to the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery.

Relevant published literature will be used as a template for experimental design and decision-making (Workman et al., 2010. Guidelines for the welfare and use of animals in cancer research. BJC, 102, 1555-1577).

We will follow guidelines of good practice [Morton et al., Lab Animals, 35(1): 1-41 (2001); Workman P, el al. British Journal of Cancer, 102:1555-77 (2010)]. Administration of substances and withdrawal of blood will be undertaken using a combination of volumes, routes and frequencies that themselves will result in no more than transient discomfort and no lasting harm.

Aging mice will be monitored and managed according to Wilkinson et al (2020) Laboratory Animals: 54(3): 225 – 238.

We will consult the NC3Rs guidelines and monitor refinement where such practices are published (NC3Rs website and elsewhere).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By reading 3Rs literature and participating in 3Rs workshops locally and nationally; attending annual Licensees meeting at CRUK-MI. Through discussing refinements with our NACWO, NVS and ASRU.

69. Ecology and evolution of pathogen- microbiome-host interactions during population-level intermingling

Project duration

3 years 0 months

Project purpose

- Basic research

Key words

Cattle, coronavirus, microbiome, infectious disease

Animal types	Life stages
Cattle	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 better understand the effect of commingling of calves from different source farms on the transmission of calf commensal bacterial populations and coronavirus located in the nasal cavity and faeces.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Commingling is associated with increased infectious pathogen transmission risk with possible global consequences, as the COVID-19 pandemic has highlighted. This work will help us to better understand the roles of the host and host-commingling on transmission of pathogens, such as coronavirus, and commensal bacteria. This will enable us to develop better interventions to reduce pathogen spread among populations.

What outputs do you think you will see at the end of this project?

Outputs will include:

A greater understanding of the role of population changes on the sharing of commensal bacteria and pathogens

Several publications in peer-reviewed open-access journals

US-UK knowledge exchange

Who or what will benefit from these outputs, and how?

Beneficiaries include:

Livestock: the knowledge outcomes will better inform livestock husbandry and management to improve health and welfare

Academics: new knowledge shared and research partnerships developed

Farmers: improved livestock health and welfare and subsequent improvements in job satisfaction, well-being, and business profitability

How will you look to maximise the outputs of this work?

Several publications in peer-reviewed open-access journals regardless of study outcomes

US-UK knowledge exchange among multiple institutions

Presentation of findings at scientific conferences and farmer meetings

Sharing of new knowledge with the livestock industry via various media outlets

Species and numbers of animals expected to be used

Cattle: 150 weaned calves will be used for the 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.

Bovine calves are at greatest risk of respiratory disease after weaning, which is why it is necessary to study this age group. This is due to a number of factors: stress of commingling with unfamiliar calves as weaning typically coincides with batching of calves into larger groups; waning maternally-derived antibodies and an immature immune system; and, stress associated with dietary change.

We are choosing to specifically study bovine calves for multiple reasons:

Bovine calves experience a high incidence of respiratory disease especially after weaning.

Calves are large enough to obtain nasopharyngeal samples unlike smaller rodent models.

The outcomes of this study will have cross-species relevance but will be immediately relevant to the livestock industry and will help inform improve respiratory disease mitigation efforts.

Typically, what will be done to an animal used in your project?

Project duration: 5 weeks per cohort

Length of stay on premises: up to 12 weeks per cohort, but most likely less than 8 weeks. Calves will be sold at the end of the study.

Accommodation: Calves will be housed in groups of at least two calves throughout the project. Calves will be provided with sheltered area that has ample bedding (such as straw) to comfortably lie down. Calves will have an unobstructed floor area of at least 1.5 m^2 per calf, which is the minimum statutory industry requirement for calves < 150 kg (Statutory Management Requirements 11: Welfare of Calves). This space allocation is justified because we are studying the impact of intense commingling on calf-calf transfer of bacteria and coronavirus and so need to provide the lower permitted space requirement to replicate this intense commingling. Calves will always have access to water and food appropriate for weaned calves (a mixture of concentrates and forage). Fresh feed will be provided daily. Fresh bedding will be provided as needed to keep the bedding surface dry and clean. Calves will be housed on a free-draining area to reduce moisture accumulation in the bedding. The housing area will have an overhanging roof to keep the calves and bedding dry.

Source of calves: Weaned calves will be purchased from up to 10 commercial dairy farms. These farms will be selected based on herd size as they will need to be large enough (> 200 milking cows) to provide a sufficient number of calves during the study periods. Calves will be transported in a livestock trailer according to the Animal Welfare Act 2006.

Calf body mass: Calves will be weighed on arrival and periodically thereafter to monitor growth rates.

Calves will undergo the following procedures:

Nasopharyngeal swabs

A soft and flexible proctology swab will be inserted into each nostril and gently guided to the back of the nasal cavity. Once there, it will be rotated several times for 3 seconds and then removed. This procedure will be performed 5 times throughout the study.

Faecal sampling and rectal temperature

Calves commonly pass faeces after a thermometer has been placed in the rectum for sufficient time to obtain an accurate temperature; therefore, a rectal temperature shall be

obtained first and a free catch faecal sample collected where possible. If the calf fails to defecate within two minutes of the thermometer removal from the rectum, faeces will be collected from the rectum using a gloved finger. Fecal sampling will be performed 5 times throughout the study.

Blood sampling

Venous blood samples will be collected from the jugular (neck) veins. Up to 40 mL of blood (four 10 mL blood collection tubes) will be collected on each occasion on up to 5 occasions throughout the study (as detailed in the Protocol section).

All of the above procedures will be performed five times and no less than 24 hours apart.

Restraint for procedures: Calves will be restrained manually for all procedures. One person will restrain the calf whilst a second person obtains the samples. Calves will be restrained for a maximum of 5 minutes, which provides sufficient time for all of the procedures listed above to be performed.

What are the expected impacts and/or adverse effects for the animals during your project?

Calves will be somewhat stressed during the brief periods (up to 5 minutes) of restraint necessary to perform the minimally invasive procedures that this study involves.

Calves are expected to experience mild and transient discomfort during the swabbing of the nose. There is a small risk of causing a nose bleed because the back of the nose is highly vascular. Like a nose bleed in humans, however, the bleeding will only last a short time (up to several minutes) and the calf will experience little, if any, pain in association with the nose bleeding.

Calves will experience momentary pain associated with blood sampling. Blood sampling 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.

Faecal sampling will cause the calf to experience transient discomfort if the sample is obtained manually. There is a small risk of injury to the rectal mucosa associated with using a rectal thermometer. This typically occurs when an animal is inadequately restrained; consequently, calves will be restrained manually by a second person. A free catch sample will not affect the calf.

Other events that may cause momentary distress in this study include transport, mixing with other calves, and the compulsory pre-movement TB-testing. These events are likely to cause minimal stress and are typical farm husbandry practices.

The mixing of weaned calves from different source farms following weaning is a common industry practise and will be performed as part of our project. We expect that this will increase the risk of calves developing infections, particularly respiratory pathogens, as this is a known risk factor. We do not anticipate, however, that the infections will cause severe disease as they will not be exposed to additional stressors, such as malnutrition or poor

housing, that would cause sufficient immunosuppression to worsen the severity of the respiratory disease. We anticipate that the severity of any disease, if infected, will be similar to children developing coughs and colds following exposure to classmates after a holiday. Calves will be monitored daily for signs of ill health and treated accordingly as described elsewhere.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Calves: Mild severity (100%) for everything except infectious disease, which is mild to moderate severity (proportion unknown as not a controlled aspect of the study).

What will happen to animals at the end of this project?

- Kept alive
- 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 to understand how the commingling of animals affects the transmission of bacteria and viruses among animals and, consequently, alters an animal's commensal bacterial population or microbiota. Controlled experimental studies using animals are essential because this is a complex process that involves social interactions, the host immune system, and an animal's commensal bacterial populations. We do not know enough about these various factors and how they interact to answer our study question without using animals.

A search of three databases (PubMed, Web of Knowledge, Google Scholar) indicates that our proposed study has not been undertaken before. Other studies have, however, investigated nasal and faecal bacterial populations, but they have not investigated how commingling alters these bacterial populations.

Which non-animal alternatives did you consider for use in this project?

Computer modeling can be useful where sufficient information is known to predict what is likely to happen, but it does not explore the underlying causal mechanisms.

Epidemiological data from human and veterinary populations. This has helped us develop our hypotheses and shape our project design.

Lower-order invertebrates will also have their own commensal bacterial populations, but they are too far removed from the complexity of higher-order animals (different immune systems and social structures etc.)

Why were they not suitable?

Computer modeling.

Computer modeling is useful when modeling infectious disease transmission in populations when we have sufficient knowledge of the pathogen and host populations, but it is not useful when we do not know the fundamental drivers of bacterial transmission during commingling events. This has not been sufficiently studied to provide basic information needed for modeling. We expect that computer modeling will be useful in the future when we have a greater understanding of the fundamental processes.

Epidemiological data

Data from human and veterinary epidemiological studies have been used to inform the development of this project. This information, however, is only enough to tell us what is likely to happen but it can not tell us what will happen or why.

Lower-order invertebrates

To understand the impact of commingling on the transmission of respiratory and gastro-intestinal bacterial populations among hosts, we need to use animal species that has complex social behaviours and are large enough to facilitate the collection of 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?

The least number of animals needed to achieve our project goal has been chosen with the help of expert opinion and advice from statisticians. This is based on the four commingling categories varying in the number of source farms (SF) and relative abundance or evenness (E) of calves from each source:

Homogenous: single source (commingling negative control)

Low SF, Low E: 2 source farms with 1 compromising 80% of calves in the pen

Low SF, High E: 3 source farms, each with ~33% of calves in the pen

High SF, High E: 5 source farms each with 20% of calves in the pen.

Each of the four commingling groups will be replicated three times for a total of 12 pens throughout the study. Up to 10 calves will be assigned to each of the four pens, for a total of 120 calves across all three cohorts. Up to ten calves are needed per pen to provide sufficient robustness should some of the calves require removal from the study. For example, if one calf develops respiratory disease in Group 4 (High SF, High E), we can still maintain 5 source farms if we have two calves per source farm. This is more important than the impact on the relative abundance of calves from each source farm. We also need to account for some calves becoming ill during the initial two-week quarantine period (anticipate up to 10%) and requiring antibiotic treatment. Calves treated with antibiotics will be ineligible to participate in the remainder of the study. Therefore, up to 50 calves will be brought onto the University farm for the project for quarantine, but of those, up to 40 calves will be used for the study; consequently, we require 150 calves in total for the project so that we have at least 120 healthy calves available for commingling at the end of the quarantine period. The remaining 10 calves per cohort (30 calves in total) will be housed separately from the project calves.

We anticipate that the calves from the different source farms will have nasal and faecal commensal bacterial populations that are more similar to calves from the same source farm than calves from different farms. Our calculations indicate that we will be able to characterise the impact of commingling for calves from different source farms on the commensal bacterial populations using the numbers in this study. These calculations are based on studies of calf commensal bacteria populations and variability. Data does not exist from studies of animal commingling to inform design, which is why we are undertaking this project.

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 will source calves from different farms. Based on prior research studies, we anticipate that calves will have commensal bacterial populations similar to calves from the same farm. By using farm- level similarity in host bacterial populations we can use fewer animals to study the effects of commingling than if all the calves were sourced from the same farm.

Due to the complexity of the calf commensal bacterial populations being studied and modeled, we sought expert opinion from collaborators in the USA and specialist statistical advice during the study design process to determine the number of calves needed to meet the outcomes of the project. Online tools, such as the EDA NC3R's, were evaluated but did not sufficiently address our project needs.

Calves will be vaccinated on arrival at the University farm to reduce the risk of respiratory disease.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Calves will be sold at the end of the project by private sale or auction market back to the commercial livestock industry.

All data generated from the 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.

Weaned bovine calves will be studied using the most minimally invasive procedures needed to meet the project aims for mulitple reasons:

Calves are commonly affected by respiratory post-weaning in association with commingling and so provide a natural model of pathogen transmission. The new knowledge resulting from our study will help mitigate respiratory disease in the cattle industry by better informing control methods.

Dairy calves are accustomed to regular human contact as they are typically separated from their mother at birth and cared for by farm staff. This means that they will find it less stressful than an equivalent-sized large animal model.

Calves are sufficiently large enough to obtain the required samples (nasopharyngeal swab) without having to euthanize the animal, as may be the case for a smaller animal species.

Swabbing the nasopharynx and collecting faeces (for microbiome and viral detection) and blood sampling (for immune response evaluation) are the most minimally-invasive methods needed to obtain the data required to meet the aims of this study. The calves will experience transient discomfort during the sampling and up to several hours after (bruising due to venipuncture) but will make a full recovery and will be sold at the end of the project.

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 do not have the complex social structure require to study the impact of commingling on pathogen transmission

They are too small and do not have the organ structures, such as the nasopharynx, needed to obtain samples.

The animals have to be obtained from disparate sources, which may be more complicated for other species. Dairy calves can be traced to the farm of origin.

Less sentient species do not have their immune system is too different to provide meaningful results.

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.

70. Evaluation and treatment of cognitive and neuropsychiatric changes in animal models of ageing and dementia

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Alzheimer's disease, amyloid, therapy, cognition, genetic modification

Animal types	Life stages
Mice	adult, aged, juvenile, pregnant, neonate
Rats	juvenile, adult, aged, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the brain’s cellular and systems mechanisms that cause age-related cognitive and neuropsychiatric changes using rodent models of aging and dementia.

To use this information to test interventions (biological and environmental) to inhibit and/or reverse brain changes and improve cognitive/neuropsychiatric symptoms in rodent models of cognitive decline in ageing and 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?

As medical research and care continue to prolong human life, the incidence of age-related cognitive disorders, are increasing. The current number of newly diagnosed individuals with dementia in the UK is 210,000 (Alzheimer's Research UK). Globally the number of people living with dementia is approx. 50 million and is predicted to rise to 152 million by 2050 (c.f., https://www.dementiastatistics.org/statistics/prevalence-projections-in-the-uk/). The financial cost of dementia in the UK is predicted to rise from £26bn to £55bn by 2040 and may be considerably higher given recent economic events (Alzheimer's Research UK). Given the rise in the incidence and costs associated with dementia care, it is disparaging to note that there is no effective disease modifying treatment for dementia (other than symptomatic and relatively short-term interventions). A treatment that can slow or prevent the onset and progression of dementia by as little as 5-10 years, could push disease onset outside the normal life expectancy range. Such an advance would result in substantial financial and psychological benefits to those at risk of the disease and by implication those involved in the care of patients.

Without question, the most effective treatment for dementia can be achieved during the prodromal phase of the disease; that is, before substantial brain cell loss and core clinical life-changing symptoms emerge. However, identifying early brain and cognitive changes remains a challenge and this has held back the development of appropriate treatments. There remains an urgent need to understand and identify the genetic and earliest biological and cognitive indicators of dementia to aid diagnosis and develop therapies that target appropriate biological mechanisms, in what is a dynamic disease process.

This project has two aims:

To use animal models of dementia-related brain pathology to understand the link between genetic and environmental risk factors for dementia, their associated brain changes and mechanisms, and their impact on behaviour (e.g., memory loss and emotional changes). To achieve this, the project will use current state-of-the-art genetically modified rodent models, contemporary neuroscience and behavioural methods to characterise pathological processes, their associated brain changes at the cellular and systems levels, together with resultant cognitive and neuropsychiatric disturbances.

To evaluate putative therapeutic interventions that target early-stage dementia-related pathological processes. Examples of such interventions will include modification of the brain renin- angiotensin system (RAS) and the use of ultrasound to modify neuronal function and pathological processes. Where possible, we will combine this work with research on human brain tissue that will serve to test the validity and potential translation of our work.

What outputs do you think you will see at the end of this project?

We will produce publications in relevant journals and submit grant applications based on our new findings. We will work with academic and industrial partners to evaluate novel therapeutic approaches to facilitate translation to clinical applications. We will also liaise with professional and lay representatives of charitable organisations, such as Alzheimer's Society, to ensure our findings reach a range of different communities and service users.

Who or what will benefit from these outputs, and how?

In the short term, the beneficiaries will include the wider academic/scientific community, research staff and students.

In the longer term, our findings may inform policy regarding the identification and use of biological, cognitive and behavioural measures associated with early-stage AD. For example, our work on the brain RAS systems may be beneficial in repurposing hypertensive drug medication for those at risk of developing dementia. Our longer-term aim is that our research will benefit patients and those at risk of dementia.

How will you look to maximise the outputs of this work?

We will maximise the output and impact of this work through collaborations that will involve multiple levels of analysis and the use of the most up-to-date scientific techniques (e.g., in vivo Ca2+ imaging and ultrasonic transcranial stimulation). This strategy will promote the efficient and effective use of animals. This strategy should allow us to realise publications in well-respected and high-impact journals and secure further grant funding from government and industrial sources. Where null results are obtained, we will maximise their impact by using statistical approaches (such as Bayes analysis) to confirm the robust nature of the observation and their theoretical significance. This will help in reducing the need for further replication. Where appropriate, we will use pre-registered reports (https://www.animalstudyregistry.org/asr_web/index.action) and adhere to ARRIVE 2.0 guidelines. New knowledge will be disseminated rapidly through traditional means (publications and online media).

Media communications experts in the establishment will ensure that any novel findings are communicated to relevant parties. We have an active network of AD carers who visit the laboratory and we will ensure that our findings reach the local AD carers community. We will also present our work at local (e.g., UK Alzheimer's Society) and at international meetings.

Species and numbers of animals expected to be used

- Mice: 3000
- Rats: 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.

This project will use rodents (mice and rats) to study the brain mechanism underpinning cognition and neuropsychiatric symptoms associated with dementia and age-related cognitive decline. The project will use genetically modified rodents that possess human genes or manipulation of genes linked with core pathological hallmarks of dementia (e.g., rodents with amyloid or tau genetic manipulations and mouse models with a direct link to dementia disease processes, e.g., Down syndrome mouse models, as patients with this condition often show early-onset Alzheimer's disease), or manipulation of genes identified

for example by human GWAS studies as conveying risk/protection for dementia. In some cases, mice expressing genetic modifications that allow manipulation of specific classes of neurons will be used to identify the networks disrupted by pathological changes during aging. Aging is a major risk factor for cognitive decline and the onset of neurodegenerative conditions. Therefore, the project will use rodents of both sexes across the lifespan to explore how aging (and gender) interacts with disease onset and progression. Rodents are most appropriate for this project because the technology exists to manipulate their genes, and there is a large body of literature detailing the content, conditions and mechanisms of emotion, learning and memory in rodents. Rodents age relatively quickly (typical life-span is up 18 months) and represent an efficient and valid model of age-related brain and cognitive changes that are observed in humans.

Typically, what will be done to an animal used in your project?

One aim of the project is to characterise rodent models of dementia, to determine the relationship between changes in genes, behaviour and pathology that in turn can inform understanding of the impact of drugs (or other interventions) on disease processes and ultimately cognition. Rodents with genetic modifications or wild-type controls will be bred and maintained at the facility before being transferred to behavioural testing before or after /or surgical/drug procedures. In a typical experiment, rodents possessing genetic modifications l (such as mutant human amyloid precursor protein (APP) mutations; e.g., $APP^{NL\text{-}G\text{-}F}$ mice or rats), and associated wild type control animals, will undergo behavioural testing at specific points during aging (for example, 3 months, 6, 10 and 16 months of age). Behavioural testing may involve spontaneous exploratory behaviours such as interacting with object in an open field, learning to solve puzzles such as multiple choice mazes or navigating to a hidden location (e.g., in a watermaze), operant or classical conditioning procedures to investigate associative memories. Animals will be motivated to problem solve using access to food or water rewards. Mice will not be subject to food motivation to avoid complications associated with the the risk of torpor in this species. Memory decline is one of the earliest cognitive changes seen in dementia patients, we will use behavioural tests to track the nature and extent of memory changes in rodent models to determine how drug or other interventions can be used to slow or stop progression of pathology and cognitive decline. Tasks will be chosen to map onto theoretically relevant brain networks, such as the hippocampus, amygdala or frontal lobes, that are impaired in patients. In some experiments we will use mild electric footpad stimulation to promote rapid encoding of emotional memories. This procedure engages well-established and tractable changes in brain networks that are relevant to memory and neuropsychiatric changes in aging and dementia, such as anxiety and depression. Alternative procedures, such as air puff conditioning or instrumental avoidance procedures, do not engage the same brain networks and may introduce additional variability or confounds that require increased number of animal to achieve robust statistical analyses. The foot shock protocol will be used to study the acquisition and consolidation (storage) of memory and subsequent retrieval and re-consolidation of event memory during the course of disease progression. This will inform our understanding of how different stages of the disease impact core psychological and biological processes critical for normal memory and retrieval. The animals will experience very brief mild pain and discomfort with exposure to very brief periods of footpad stimulation. We are committed to refining our procedures and will explore ways to reduce exposure to aversive events (e.g., reduce intensity or number of foot shocks), e.g., by taking advantage of strain differences in learning rates, while meeting the scientific aims of the project. Cognitive changes are commonly recognised as a feature of dementia. However, neuropsychiatric changes can both precede and develop

during dementia progression, such as depression, anxiety, apathy and agitation. These features of dementia have not received as detailed scientific attention as memoryu dysfunction and their relationship to progressive brain pathology and sensitivity to putative treatments remains unclear. For example, depression in dementia is often resistant to standard anti-depressant SSRI medication, e.g., sertraline, fluoxetine, and such treatments may be harmful. We will use longitudinal experimental designs to monitor spontaneous changes in mood and depressive behaviours. For example, we will examine food preferences to measure anhedonia, exploratory activity and problem solving tests to examine anxiety and apathy in rodents, respectively. These tests may be repeated at key stages of development to track the time-course and nature of behavioural changes. Some rodents may receive interventions (e.g., drugs) designed to alleviate symptoms and or modify disease processes.

In addition to behavioural testing we will examine more directly how dementia pathology alters brain activity to understand the disease process and the impact of treatments. We will use techniques such as in vivo electrophysiological recording of cells or two-photon calcium imaging to record (and quantify) neuronal activity during learning experiences. The latter experiments will use rodents at specific stages of pathology and will last no more 3-4 months. The in vivo recoding techniques will involve implantation of devices under general anaesthesia using stereotaxic methods to place equipment in theoretically relevant brain region (e.g., the hippocampus and ventral subiculum). During surgeries, very small windows will be made in the skull to gain access to the brain, once implanted a device will be held in place with tiny screws (no more than 4 mm long), secured in place with dental cement before closing the wound. In some experiments, following a mircoinjection of substances to reveal changes in calcium activity in neurons, a small glass window will be attached to the skull to allow subsequent imaging of neurons using a two-photon microscope. Animals will experience mild, transient pain that will be limited by the use of appropriate anaesthesia and analgesia. None of the procedures will impact the special sense or maintenance behaviours. Drug administration will typically be achieved via drinking water or other appropriate routes, such as i.p. or sub-cutaneous injections. Typically, animals will experience mild and transient pain but no lasting harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal). Where administration is required for prolonged periods or directly into core brain regions, animals may surgically implanted with a cannulae or slow release devices, such as a mini-pump. These animals will experience some discomfort after surgery and some mild to moderate pain that will be treated with analgesics. In some experiments, animals will undergo a restriction of access to food or water (to no more than 80% of their free-feeding weight) to motivate them to solve tasks. This is not expected to cause distress and reflects a similar motivational drive encountered in the wild. In some experiments, animals will experience mild and transient restraint anxiety from handling, e.g., blood sampling or measurement of tail blood pressure. The animals will be slowly and progressively acclimatised to the restraint to reduce distress.

We also intend to examine the impact of focussed ultrasonic brain stimulation (FUS) on brain activity, cognition and pathology in rodent models of dementia at key stages of disease pathogenesis. There is evidence to show that ultrasonic energy can have a facilitatory or inhibitory effects (depending on parameters) on neural function, as well as promote localised changes in the blood brain barrier (the latter could facilitate targeted delivery of drugs (such as antibodies) to specific regions. We will use this technique to determine whether FUS can reduce or help eliminate AD pathology or counter deficits in synaptic plasticity that underpin some of the cognitive changes in the early stage so

dementia. This procedure will be conducted under general anaesthesia and the animal may experience mild distress/discomfort but no pain. This is a non-invasive technique that requires no direct surgical intervention as sonic energy is projected onto target tissue through the skull. The experiments will last typically 3-4 months and may involve several sessions of FUS, under general anaesthesia, in order to track the impact of the intervention on disease progression and behaviour. We are committed to further refining these procedures and will assess ways to, e.g., reduce exposure to anaesthetic, modify the properties of untrasonic stimuli, to achieve our scientific aims and reduce distress to the animals.

At the end of the experiments, brain tissue will be collected after the animal has been killed via a Schedule 1 method and biochemical analysis and histological analyses carried out to establish the links between genes, interventions, and cognitive changes. 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 but no pain.

Wherever possible the order of tests will be counterbalanced (to minimise the influence of cumulative experience and other extraneous variables) and, where possible, we will use within-subject designs to reduce variability and the number of animals. Techniques and their combination will be selected in a manner to minimise distress to the animal and avoid cumulative severity. For example, animals will not be subject to a combination of different tests using aversive stimulation (e.g., watermaze and electric footpad stimulation). Data analysis, e.g., of object contact times, will be conducted by computer where appropriate, the experimenter will be blind to experimental conditions or evaluated by independent observer for accuracy and rigour. Control animals will be used to establish the normal profile of brain and/or cognitive changes associated with age and provide a reference condition for statistical analysis. In all cases we will seek to refine our procedures to redeuce animal distress and take note of advances in behavioural and biological techniques.

What are the expected impacts and/or adverse effects for the animals during your project?

None of the genetic and brain manipulations are designed to interfere with the special senses or negatively impact the animal's maintenance behaviours, e.g., eating and drinking. When genetic mutations in rodents are used to mimic dementia pathology, the cognitive changes are typically progressive, age-related but do not reach a point where the animal is incapacitated, develop motor problems or where social and maintenance behaviours or the special senses are affected.

In studies where ageing is a critical factor, the animals may be maintained in the laboratory typically for approximately 15-16 months of age. However, in some circumstances where the biological changes accrue more slowly with age (typical for late onset dementia in humans) we may maintain animals for for up to 24 months of age. All animals will be systematically monitored for signs of ageing-related health issues (e.g., abnormal weight loss, tumours etc). They will be maintained in standard group housing with environmental enrichments, such as nesting material. Other experiments may age animals up to a specific age point (e,.g., 6 months of age) to study the effects of pathology and interventions on cognition, after which brain tissue will be collected for analysis.

Drug administration will be via the most appropriate route to cause minimal distress (e.g., in drinking water or i.p. injections). But the animals may experience stress whilst being handled, but to reduce this as much as possible, the animals will be habituated to handling before the procedure is carried out.

Some animals may receive surgical manipulations that will involve direct intervention with the brain. This will be carried out under general anaesthesia with appropriate analgesia to manage post- operative pain. The animals are expected to recover quickly and engage in normal maintenance, social and learning behaviours just as patients recovering in hospital. Animals will typically be used for 3-4 months post-surgical intervention.

One of the major outcome measures of project is the animal's natural behaviour when confronting environmental challenges, such as navigation and problem-solving and thus we wish to minimise the impact of the procedures on basic sensory/motor and motivational processes allied to the performance of these key outcome measures. The experimental manipulations are designed to result in subtle changes in cognitive function, (e.g., subtle alterations in memory and attention, reaction to novelty, reactions to stimuli paired with aversive events) or improve aspects of cognition.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Protocol 1 :

Mice: 40% mild, 60% moderate

Rats: 40% mild, 60% moderate

Protocol 2:

Mice: 80% Moderate (20% mild)

Rats: 80% Moderate (20% mild)

Protocols 3 & 4:

Breeding and maintenance of GA: all mild. Protocol 5:
Maintenance of genetically modified rats or mice up to 24 months of age: all mild.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of this project is to understand neurodegenerative processes that contribute to cognitive and neuropsychiatric changes in old age and in dementia. There is a lack of understanding regarding the nature and mechanism of dementia-related brain changes, especially during the early prodromal stages of the disease in humans. Animal models currently provide the best method for investigation prodromal brain changes in dementia. Investigating the nature of these brain changes is vital to treat the condition before major brain cell loss occurs. There is an urgent clinical need for disease-modifying therapies as currently there is no cure for dementia. Because the behavioural and cognitive changes associated with dementia are a manifestation of complex neural activity within and across brain structures, other model systems, e.g., those that do not have homologous brain structures, such as Aplysia, or cell culture model systems, cannot address the questions under consideration. Rodents share extensive homologies with humans at the neural, anatomical and psychological levels and thus provide a valuable platform to investigate complex relationships between disease, genetic/environmental risks, brain activity and behaviour. For these reasons, rodent models, continue to represent a fundamental step in the development of clinically meaningful treatments for complex human psychological disorders.

Which non-animal alternatives did you consider for use in this project?

Alternative approaches to studying neural changes with genetic mutations linked to dementia include cell culture systems, invertebrate species and mini-brain approaches. These techniques provide important insights into local changes in neural function but fail to capture network changes and how, for example, genetic risks interact with normal ageing processes to reveal dementia. We have developed collaborations with colleagues that are experts in these techniques to support our work. This will improve our understanding of disease and drug mechanisms at the cellular level and help validate potential drug targets. This knowledge can then be applied to rodent models of dementia for the reasons outlined above to provide an assessment of broader brain function and cognition. This translation between cell-based model system into an in-vivo setting will inevitably reduce the number of animals used to test compounds in the treatment of dementia. We also have close collaborations with researchers using human brain tissue that will inform our studies and ensure we tackle disease- relevant processes.

Why were they not suitable?

It is important to acknowledge that non-animal approaches can contribute to the field’s broader scientific goals. However, in isolation they cannot address the nature of brain network changes underpinning dementia and cognitive decline. Nor can they help identify how drug interventions may (or may not) alleviate disease symptoms at the biological and behavioural level. Rodent and humans numerous brain homologies and similarities in complex cognitive processes that aid in the translation of our work to clinical applications. Furthermore, because dementia is an age-associated disease, cell culture studies cannot mimic cellular biological and environment changes that occur naturally over months and years.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 calculation of the number of animals used in this project is based on considerable prior experience with breeding and using genetically altered and wild-type rodents. We intend to use both males and females in the experiments (which must be suitably powered) to detect sex-specific differences in disease or treatment responses. Using both sexes will reduce animal waste from the breeding program and ensure our experimental findings are relevant to both sexes in terms of potential clinical applications. There is an extensive literature supporting the theoretical underpinning of the research and a vast amount of data indicating the power required in specific rodent tests and these will be consulted to ensure the data is valid and robust. We will also carry out statistical power calculations (based on preliminary data) and support null results with appropriate statistical tools (such as Bayes analysis) to ensure the validity of our conclusions.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Where possible we will use within-subject comparisons to evaluate drug treatments (e.g., before vs after treatment). This method helps to reduce the impact of between-subject variability when evaluating behavioural outputs. Biochemical measures can be variable but we have selected group sizes based on our own experience/published work. We are able to use power calculations based on previous data sets to estimate the size of control and treatment groups together with information about the nature of statistical methods. For example, whether the hypothesis predicts main effects of group or interactions between treatment groups. Null effects will be evaluated using Bayesian statistics to ensure robust acceptance of the null hypothesis and thereby reducing the need for replication. Many of the refinements in the generation of recent animal models of dementia have used standardised background strains (such as C57B6 lines) as opposed to hybrid background strains. This has resulted in reduced variability and thus a reduction in the number of animals used in protocols. The majority of the genetically modified mouse lines used in this project will involve the C57B6 strain. Rat studies will typically involve Lister hooded rats or if using genetically modified rats a common background strain, such as Wistar. We have extensive experience with these rat strains and understand their cognitive characteristics, aiding in the experimental design and power of our analyses. In all studies, experimental factors, such as the order of test, the nature of stimuli (and other experimental parameters such as gender) will be counterbalanced to reduce the influence of extraneous variables on the outcomes of the experiments. Independent assessment of, e.g., behavioural outcomes, will be used to confirm and validate our data. We will avoid repetition of published results where possible unless such information in combination with our new techniques is theoretically meaningful and clearly advances our knowledge.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where feasible and economically viable will breed our experimental cohorts in-house to ensure our experiments are timed precisely and environmental conditions are consistent across animals. We will optimise the information gathered from animals. For example, by using in vivo measures of brain function (electrophysiological recordings; or two-photon imaging) together with behavioural tests and biological endpoints (histology, biochemical assays). This approach will enable us to analyse the effects of genes and/or brain changes and drugs at the molecular, cellular, network and behavioural levels (often in the same animal). We will also offer research colleagues access to surplus rodent brain tissue to aid scientific advancement in affiliated areas.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 make use of wild-type and genetically modified rodents. This will include transgenic and knock-in mice or rats that possess genetic mutations linked to dementia. The majority of studies will employ mice but rat genetic models are becoming more available and these may provide opportunities/techniques to investigate brain function relevant to the aims of the project. For example, identical genetic alteration in rats and mice (e.g., mutant APP expression) may result in different profiles of tau pathology between the species. We will therefore select the most appropriate species to address the scientific of the project. For example, if we wish to understand the contribution of marked age-related tau induced by amyloid pathology in the development of For example, idetical genetic alteration in rat For example, idetical genetic alteration in moc neuropsychiatric symptoms, we will appropriate rat models. In all cases, the genetic mutations should not influence maintenance behaviours or the special senses but result in subtle (often age-related) cognitive changes. While first generation transgenic models of dementia (such as Tg2576 mice) offered important insights into pathological processes, they have several limitations. The latest second and third generation knock-in models of dementia (such as $_{\text{APP}}{}^{\text{NL-G-F}}$) have fewer limitations, have excellent construct validity and represent a considerable improvement in modelling brain pathology.

In some experiment, the animals may undergo brief periods of food or water restriction to motivate them to learn and solve specific tasks. A deficit in food and water intake is not unusual for wild type rodents as evidenced by their foraging behaviour and their ability to adapt their behaviour to access food and water. However, research with has shown that caloric restriction in mice can lead to torpor (defined as a state of substantially decreased metabolic rate, most notably in females, Swoap & Gutilla, 2009), which can impact the animals' behaviour and brain electrophysiology (e.g., Huang et al., 2021 Sleep, 44,9,2021). Caloric restriction in rats can lead to changes in body temperature and metabolism but does not appear to lead to apathy and inactivity, characteristic of torpor in mice.

Indeed, calorific restriction in rats is associated with increased longevity (e.g., Duffy et al., 1989). There is some evidence (as reviewed by the working group on fluid restriction in rodents (Barkus etal., 2022) that food restriction may be considered the more refined approach to achieve sustained motivation.

However, they acknowledge that further research is needed given evidence that mice tolerate water control better than food restriction and this group did not consider issues associated with torpor in mice. Based on our experience and recent evidence of the impact of caloric restriction on neural function, water restriction appears to be the most refined method in mice to achieve sustained motivation. Thus, to mitigate the potential impact of torpor interacting with the effects of genetic mutations in mice on behaviour and neural function, we will employ (non-restricted)behavioural tests that index exploratory memory (e.g., object recognition paradigms). If the experiments require a level of motivation to sustain behaviour in mice during testing, we will use water restriction to promote instrumental or Pavlovian behaviours. Water restriction will ensure comparable and sufficient level of motivation to engage rodents in problem solving behaviour, levels of restriction can be easily adjusted throughout the study to accommodate growth of the animal. Furthermore, fluid rewards can be used that provide positive hedonic experience for the animals (e.g., sucrose solution). Water restriction will be applied based on the recommendation of the NC3R working group investigating fluid restriction in rodent (Barkus et al., 2022; J Neuros Method 381, 109705).

A direct aversion to food or fluids may be induced by ip administration of lithium cholride and this aversion used to assess food preference/hedonic value in rodents. This is the most refined method to ensure that the aversion is similar in duration and severity in each cohort (injections are tailored to the weight of each animal). There are alternative procedures that we have considered. For example, high speed rotation, high ambient room temperature, exposure to magnetic fields. All of these are coercive and highly aversive to rodents and increase the probability of injury to the animal (c.f., Nakajima 2018, Exp Anim 68(1) 71-79). A less aversive alternative to using lithium chloride is wheel running. Nakajima (2018) showed that exposure to wheel running after consuming a novel food exaggerated the neophobic reaction to the novel food. Furthermore, rats showed pica behaviour (kaolin clay ingestion) after wheel running, which is suggestive of intestinal discomfort. This procedure provides an alternative method to maintain a neophobic reaction. This is unsuitable for our purposes because the aversion will be used to reduce preference to familiar food/fluids (to investigate changes in hedonic and incentive value of rewards). Furthermore, wheel running is under the control of the rodent and may lead to increased within-group variability and thus the need for larger N. In some cases, genetically modified rodents may show baseline changes in wheel running behaviour, thus compromising the degree of aversion established in reference to WT control animals. For these reasons the establishment of an aversion using lithium chloride is the most refined method.

Given evidence of neuropsychiatric changes in dementia, such as depression and lethargy, we include tests for anxiety (assessing exploratory behaviours), anhedonia (preference for sweet fluids) and instrumental cognitive bias tests. Typically, animals will be group housed to maintain social structures and their cage environment will include nesting tubes and bedding and maintained at standard housing room temperatures.

Surgical methods will be used to implant devices to deliver drugs to the central nervous system (such as cannulae attached to osmotic pumps), discretely remove or alter the

activity of cells in specific brain structures (such as drugs to selectively remove cells or inhibit their activity) or record from neurons and networks during behavioural tasks, (we use head mounted devices such as Neuropixel probes). The devices are designed to be small, lightweight and will not impede the animal's natural behaviours. We will record the animals behaviour as it explores its environment (e.g., to record place cells as they explore an environment using standard pellet chasing methods) or as the animals encode or retrieve task relevant information. In some experiments we may use electrical footpad stimulation to evoke fear in an animal. These tests will be used to examine the rapid acquisition and retrieval of emotional memories to permit a temporally refined analysis of brain activity during such discrete events. This procedure will result in short-term pain/discomfort but results in no lasting harm and changes in behaviour elicited by cues associated with the aversive event dissipate with time (i.e,. forgetting occurs). We are committed to refining our procedures and will carry out pilot studies to establish the most effective methods to achieve the scientific outcomes (e.g., reducing the number or intensity of shocks for specific strains of mice; Stiedl, et al., 1999, Strain and substrain differences in context- and tone-dependent fear conditioning of inbred mice, Behavioural Brain Research, 104 (1–2), 1-12).

Surgical procedures can cause short-term pain/discomfort and this will be controlled by appropriate anaesthesia and analgesia in combination with aseptic surgical conditions to reduce risk of infection and close monitoring of the animals health and well-being.

Why can't you use animals that are less sentient?

The main outcomes from the experiments are changes in psychological processes that are related to dementia. Rodents share homologous brain structures and display parallel cognitive and emotional processes (other than sophisticated language abilities) that are result of complex brain network interactions. Such sophisticated behaviours and associated brain mechanisms cannot be modelled in other organisms, such as Drosophila or Aplysia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In all procedures, we invest time in habituating animals to handling and carrying out behavioural tasks. Our main aim is to obtain natural behaviours from animals as they engage with environmental challenges, and we have well-established protocols to ensure that this is the case. There is a wealth of historical data that often describes the procedures and parameters and appropriate theoretical interpretation of animal behavioural tests. This will help ensure that our tests target appropriate cognitive processes and help reduce animal numbers. We have an established monitoring and health scoring system to record the animal's general health during their lifespan. Anaesthesia and pain relief will always be provided during surgical procedures and these will be carried out in aseptic conditions to minimise risk of infection. Animal may require food or water restriction to motivate problem solving behaviours and animals are carefully monitored during these periods to maintain a healthy weight. We will not use food-restriction in mice because of the published issues with torpor and instead use water restriction with access to fluids with hedonic value, such as, sucrose, condensed or flavoured milk, or treats (chocolate cereal) to motivate behaviour. Most of the behavioural tasks cause no harm and engage the animal's natural foraging and problem-solving behaviours. We will use fear conditioning procedures that involve administration of a limited number of acute foot shocks. The use of this procedure

will be restricted to experiments that need to interrogate well-described neural circuitry that supports this form of learning and where time sensitive changes in the animal's associative knowledge require rapid learning and discrete changes in behaviour. We will implement any refinements in surgical or behavioural procedures through discussion with colleagues and appropriately qualified persons. Where possible we will use drugs with known dose-response curves and cause no major disruption to sensorimotor function and have no impact on maintenance behaviours.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Best practice guidance will be obtained from The NC3Rs website
PREPARE and ARRIVE guidelines on the preparation, use and reporting of animal experiments. Guidance from the Named Veterinary Surgeon
NACWOs

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will monitor scientific publications for improvements in techniques and experimental designs. Where possible we will avoid direct replication of published findings and change our plans appropriately.

We will use resources such as the NC3Rs website and the ARRIVE guidelines to stay informed about advances and procedures in using and reporting animal experiments.

Where appropriate we may seek funding support from FRAME to explore ways of reducing animal numbers to achieve some of the experimental aims (as described above).

71. Identifying actin cytoskeletal regulators of metastasis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, metastasis

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?

This project aims to identify the key proteins inside the cancer cell that drive movement using zebrafish as our model 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?

There is a driving need to find new drugs to stop cancer cell spread through the body. Currently, tumors can be treated with radical surgery, chemotherapy or radiotherapy but once the cancer has spread it is almost impossible to eradicate.
Whilst there have been a number of important advances in the development of drugs to stop cancer growing the study of how cancer spread around the body has lagged behind.

This is largely due to the complex inter-relationship between the moving cancer cell and the tissue it is moving through. There have been advances in making artificial tissues which have helped us to understand some of this complexity, but these models cannot yet fully replace for animal studies which provide a much more realistic model of cancer cells moving through tissue.

This project aims to identify the key proteins inside the cancer cell that drive movement using zebrafish as our model system. In our model we will inject a ball of human cancer cells into the zebrafish embryo and test the ability of these cells to move thought-out the embryonic body. We can manipulate these cancer cells to identify those proteins that are essential for movement. In addition, we can use our model system to test how well cancer cells can move through the embryo tissue in the presence of experimental drug compounds.

What outputs do you think you will see at the end of this project?

New information on which intracellular proteins drive cancer cell migration, this new knowledge will lead to publications and could help provide the evidence base for the development and testing of novel therapeutic compounds.

Who or what will benefit from these outputs, and how?

In the short-term this project will help to identify key proteins in a cancer cell that are responsible for helping the cancer cell to spread away from the primary tumor. This knowledge could then lead to the development of anti-metastatic spreading drugs. The results we obtain will be of interest to other scientific researchers and also to pharmaceutical companies developing novel anti-cancer drugs. The project will also in the short term help promote an in vivo system for validation of other anti-spreading targets. We will be working with cells taken from breast, prostate, ovarian and pancreatic cancer. In the long-term this project could make a significant contribution towards the development of anti-cancer drugs and thus lead to clinical patient benefit for these cancer tissue types.

How will you look to maximise the outputs of this work?

We plan to publish our findings in high impact factor open access scientific journals. We plan to present our work at scientific meetings. We will also seek to publish negative findings and methods protocols though scientific journals and online archiving platforms such as bioRxiv.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 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.

We are using very early stage embryos (2dpf) because they are optically translucent and this makes imaging cancer cell dissemination possible. The 2dpf embryos are injected with cancer cells and host the cancer cells until up to 8dpf. The cancer cells injected into the zebrafish embryo are required to move through the zebrafish tissue, will encounter the primitive immune system and be required to exit from the zebrafish vasculature to enter the tail tissue. All of these migratory behaviors mimic what happens in human disease when cancer cells metastasize from primary tumor. All procedures listed here follow a non-recovery of anaesthetized animals scheme.

Typically, what will be done to an animal used in your project?

The programme of work has three Protocols

Protocol 1 concerns the maintenance and breeding of wild type and transgenic fish from which we will derive embryos to use in the dissemination assay. The transgenic fish will be those that have been modified to be transparent such as CASPER fish. We also include provision for the cryogenic storage of sperm/gametes.

Protocol 2 concerns the use of embryos derived from the fish maintained in Protocol 1. We will inject these embryos (2dpf) in the yolk sac or duct of Cuvier with human cancer cells. The cancer cells will be cell lines derived from solid tumors; breast, prostate , ovarian ,pancreatic. Thus we are testing human cells to be a physiological as possible to metastasis in human disease. Once injected the embryos will be incubated (no more than 30 embryos per dish) at 33 degrees centigrade. This elevation in normal maintenance temperature is well tolerated by the embryos and is a standard part of the dissemination assay. The elevated temperature is required to ensure the human cancer cells remain viable. Elevation of the embryo incubation temperature to 33 degree centigrade will accelerate the embryo development and we have estimated a 5 hour acceleration based on the current literature (Carole Wilson 2012).

The injected embryos will be screened 24 hours after injection for positive xenograft of the cancer cells. If there is no xenograft the embryos will be culled by a schedule 1 method. Those with a xenograft will be maintained at 33 degrees for a maximum of 5 further days (up to 8.5dpf).

Some embryos will be exposed to water containing experimental drug to test how well these drugs inhibit cancer movement through the embryo. We have already established concentrations required to permeate the embryo. Should the drugs be deleterious to embryo viability these embryos will be immediately culled.

Protocol 3 concerns obtaining Zebrafish gametes Genetically altered fish for use in this protocol may be obtained from Protocol 1 of this project or other projects with authority to breed and maintain genetically altered fish of that type and to provide them for use on other projects. Eggs and sperm are obtained from anaesthetized fish by applying gentle pressure on/or stroking the sides of the fish. (AB/AC) Fish may be killed by a Schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

Potential impacts/adverse effects that could result from this project.

Harmful phenotype: Some fish may have the potential to develop a harmful phenotype after a certain age but in all cases will be killed before reaching that age and before onset of clinical signs, unless moved on to another protocol as continued use for a specific purpose. Animals are not expected to die because of any authorized genetic alteration.

Anesthetic: Fish that do not return to normal swimming behavior within 30 minutes after removal of the anesthetic will be killed by a schedule 1 method.

Genotyping: Infections can result from fin clipping (<1%).

Injection of xenograft human cells and/or exposure to experimental drugs: This treatment could cause undue distress as the tumor grows at 6-8dpf. However, this is not something we routinely observe (<1%).

Gamete Collection: Massaging the abdomen of fish could cause scale loss leading to a breach in the epidermis and/ or dermis leave the fish vulnerable to infection (<1%) or could cause compression damage to internal organs (< 1%).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected level of severity for breeding is mild. The expected level of severity for protocol 2 is mild – in rare instances some embryos might experience distress as the xenograft grows. If the embryo becomes distressed (change in cardiovascular function, twitching) it will be humanely culled immediately using a schedule 1 method. All other protocols are non-recovery.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Advances in matrix production and 3D organotypic modelling have helped us to understand some of this complexity of cell migration in vivo but cannot yet recapitulate tissue architecture, provide local vasculature for long distance dissemination and thus fully challenge the migration of cancer cells. The zebrafish model allows us to challenge the human cancer cells with a complex tissue architecture, requires these human cancer cells to move within this tissue and also to move in and out of blood vessels. All of these activities are performed by human cancer cells in human cancer cell metastasis. So if we

find ways to block the activity in our zebrafish model system we can translate those findings to human disease.

Which non-animal alternatives did you consider for use in this project?

We have searched the literature (PubMed) and Research Gate for non-animal alternatives using search terms such as "invasion" and "cancer metastasis". We considered 3D spheroid assays and organoid cultures and both are used in the laboratory (Lab published data and unpublished data). There are also in development 3D biomaterial scaffolds that seek to provide a complex multi cell and matrix environment. We could use human participants or cancer biopsy material. Mathematical models of cell migration could be used to predict drug responses.

Why were they not suitable?

Spheroid and organoid assays do not recapitulate complex tissue architecture nor provide vasculature entry and exit points for the cancer cells. The 3D biomaterial scaffolds are not sufficiently developed to allow testing of cancer cell dissemination, being currently used to measure only proliferation, again they do not recapitulate the complex tissue architecture and vasculature encountered by a metastasising cancer cell. It is not possible to study moving cells live in a human participant and thus the only thing we can do is look in fixed tumor biopsy tissue samples. It is very difficult to ascertain if a cancer cell was actively migrating from fixed tissues. There are no accepted mathematical models of cancer cell dissemination that can be used to replace in vivo studies. Indeed, more experimental data is needed to help develop these models in the future.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For our breeding program we have estimated 1000 animals over 5 years based on a renewal of stock once a year with 6 tanks (4 tanks of adult animals and 2 tanks of juvenile fish) at any one time. 40 fish per 8L tank.

For our dissemination assay we estimate that over 5 years we will inject a large number of 2 day post fertilisation embryos, approx 2,400 per year. However of those 2,400 most will not be taken past 5 days post fertilisation and are therefore inside non-regulated embryonic age. Based on our previous 5 year project licence experience, and our efforts to refine the protocol by testing cell lines in vitro prior to embryo injection, we anticipate that 240 embryos per year will enter regulated age; this is a reduction on our previous project licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our experimental design is based on established protocols and online design assistants that we are using the optimal numbers of embryos to deliver statistical significance in our assay. We have checked our methodology with a statistician prior to publication. We always have two groups control and experimental and embryos with a positive xenograft 24 h post injection are randomly assigned to each group (20 embryos per group) - this sample size is based on a minimum differential of 5% in the dissemination score (% of embryos in each group with positive dissemination) with a 0.05 alpha value and 90% power.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Most of our work will be conducted in embryos up to 4dpf and since our previous licence application we have screened cancer cell lines for those that disseminate more quickly to reduce the number of embryos that need to enter regulated age. Wherever possible we will use these cell lines. Human cancer cells lines are a good model to use to recapitulate human disease. These cell lines have the same genetic backgrounds as found in patients and are routinely used in cancer research to identify potential therapeutic targets. However, some cell lines eg breast cancer cells do require longer incubation to disseminate and this does take us past non-regulated embryonic age. Based on our studies we now know that the best imaging window is 24 hours post injection to 48 hours post injection. This means we are imaging embryos that are 3-4 dpf - even with the additional aging due to incubation at 33 degrees (+5 hours); thus, imaging of embryos is outside of licence requirements. In certain circumstances we do need to take our xenografted embryos beyond 5dpf when a human cell line takes longer to disseminate. These embryos will be imaged up to 8dpf. In the dissemination assay we check all embryos that have been injected to confirm a xenograft 24 hours after injection. We only take forward those embryos that are positive. All negative embryos are killed by a schedule 1 method. The screening of embryos takes place at 3 days post fertilisation so is in the non-regulated window but does help to limit the number of embryos that are taken through to the regulated time frame where the cell line injected needs the longer incubation period. In our breeding programme we will endeavor to have efficient breeding and reduce surplus animals. We will consider if there is “down time” before the zebrafish population will be needed again (in-house or by collaborators) and assess the relative cost of low- rate tick-over versus archiving/rederivation. We also consider any strain- specific technical factors that may influence the number of animals required to complete the archiving/recovery process. We will use sperm freezing to archive zebrafish lines not currently 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 are using zebrafish embryos to perform cancer cell dissemination experiments. There is a mild level of severity in the experiments performed in zebrafish and we have refined our experimental protocol to use as few regulated embryos as possible. For administration of cancer cells, we determine the most appropriate cell line to use based on in vitro data from time-lapse imaging of migration in 2D and 3D. In this way we already know the cells are capable of intrinsic migration and we know that they achieve a migration speed +2um/min that will deliver the required level of migration for dissemination to the zebrafish tail. We inject the 2dpf embryos (unregulated) either in the yolk sac or duct of Cuvier and inject 100-200 cells per embryo. All injections are performed under anesthesia to reduce pain and distress. The embryos are checked after 24 hours for a positive xenograft and no adverse effects.

Why can’t you use animals that are less sentient?

We are using embryos at 2 days post fertilisation (prior to regulation), we unable to use any younger embryos because they are not developed enough for xenografting of cancer cells. We cannot use terminally anaesthetized embryos as we need the embryo host to be alive during the cancer cell dissemination to maintain tissue architecture and blood flow in the vasculature.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Protocol 1 : We will monitor our fish stocks carefully. Some fish may have the potential to develop a harmful phenotype after a certain age but in all cases will be killed before reaching that age and before onset of clinical signs, unless moved on to another protocol as continued use for a specific purpose.

Fish exhibiting any unexpected harmful phenotypes will be killed by a schedule 1 method, or in the case of individual Fish of particular scientific interest, advice will be sought promptly from the Home Office Inspector.

Protocol 2: Injection of xenograft human cells and/or exposure to experimental drugs may cause undue distress as the tumour grows at 6-8dpf. Embryos taken into regulated age will be closely monitored twice a day. If the treatment interferes significantly with locomotion, respiration or cardiovascular function; or causes significant behavioral or other physiological abnormality, then the animal will be killed humanely, without delay.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I am following established breeding protocols approved by King's College London. The dissemination assay is a published procedure from the laboratory and we are following an established protocol https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6234738/.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The principal investigator receives the National Centre for the replacement, refinement and reduction of animals in research (NC3Rs) newsletter via email. The institution Biological Services facility holds seminars/training sessions focused on 3Rs which is attended and there will be more in the future. The The Biological Services facility also disseminated information via email to all project Licence holders about 3R related policy. The principal investigator will be involved in 3Rs discussions with colleagues/collaborators at lab meetings/departmental meetings/ conferences; as well as attending seminars/events covering 3Rs topics, including those held external to their institution. The investigative team will perform regular literature searches for 3Rs advances in in the cancer research field. The investigative team will keep abreast of Zebrafish Husbandry Association and Zebrafish Information Network (ZFIN) events that are relevant to the work.

72. Identifying proteins that influence central nervous system (CNS) development and ageing

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Primary neurons, Neural development, Neural ageing, Neural regeneration, Neural repair

Animal types	Life stages
Rats	adult, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The Aim of the project is fundamental and discovery based: To identify and quantify proteins expressed during central nervous system development 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?

Research conducted under the licence will generate new knowledge that will help scientists understand how the nervous system forms and functions, and offer new insights into changes in brain that increase the chances of age-related conditions (such as Parkinson's or Alzheimer's) or how to regenerate neurons if they are damaged or die. More specifically, the research conducted under this licence will take post-mortem samples from parts of the central nervous system (CNS) of rats to unveil, for the first time, what proteins are made by groups of neurons at several stages of the animal's lifespan. This research will generate new data on what molecules ("proteins") neurons are making when

they are developing, functioning, and ageing, and will provide fundamental information for scientist to gain insights into "what proteins are neurons making when they develop, function and age?".

Neurons develop very precise connections in the brain and have very specific functions when the nervous system is fully formed. As the nervous system ages, neurons begin to work less well or even degenerate, sometimes in a very region specific manner. Scientist don't yet know how neurons are able to develop and function early in life, and what changes in them as ageing takes hold that disrupts their functioning or causes some of them to die.

Scientist don't have any pre-conceived notions of what proteins neurons are making during these stages of the lifespan, nor what different types of neurons make that enables each group to form specific connections, perform specialised tasks, or become vulnerable to particular brain conditions. Scientist currently cannot "guess" which, among more than 47,000, of these proteins are heightened at each of these life stages, so discovery-based work (such as that carried out under this licence) must first be conducted to identify and quantify what neurons are producing at each stage.

What outputs do you think you will see at the end of this project?

Publications: It is expected that the fundamental information discovered from the research conducted under this licence will be published in peer-reviewed journals and / or presented at relevant scientific conferences. Publication of the research will adhere to either Route 1 or Route 2 of UKRI Open access policy (i.e., the research will either be published within a journal that is immediately open access, or the pre-publication version of the manuscript will be available on a publicly accessible depository). Publication of the research findings will adhere to all the “ARRIVE Essential 10”guidelines for research manuscripts that use animals as a source of information. In addition to these fundamentals, the project will also adhere to the ARRIVE guidelines related to “Data Access”, as the project will generate a body of data useful to the broad research community. All data from the project will follow the “FAIR” guidelines detailed by ARRIVE (i.e., findable, accessible, interoperable and re-usable). ARRIVE’s guidelines on public accessibility aligns well with standards long established by many proteomics journals that require the deposition of the raw dataset in a open access form with a link provided in the publication. Many proteomics journals, for example, require deposition in data bases highlighted in ARRIVE guidelines (e.g., Figshare and NCBI), but also may have their own publicly accessible database deposition sites. These will continue to be adhered to when presenting the findings of the research conducted under this project.

New Information: This discovery-based project will generate new fundamental information on the protein composition of neurons in the brain during development and ageing, as well as information that could be applied downstream for treatment purposes. Because scientist don't yet know which among +47,000 proteins are made by neurons at different life stages, this project will provide compendium of the protein composition of neurons at times when they are developing, functioning or ageing. This will produce new insights into: how neural cells grow and could be regenerated (e.g., after a traumatic injury); how different neurons function differently (to give the brain a range of abilities); and why some neurons die when we age. This information will provide future hypotheses into the source of maladies affecting the CNS such as spinal cord injury, stroke, Schizophrenia and a

range of mood disorders, Parkinson's and Alzheimer's disease, as well as normal age-related cognitive decline.

Who or what will benefit from these outputs, and how?

The information generated under this licence will have significant stand-alone value, as well as generate new knowledge that will enhance subsequent research in neuroscience.

The short-term benefit will be the generation of a compendium of proteins that appear in specific neuronal cell types (e.g., motor cortex, basal ganglia, hippocampus, frontal cortex) at particular stages of life in the central nervous system. This will directly impact the knowledge base of the lab and other research scientists within the neuroscience field, by providing a starting point for research seeking to explain how the central nervous system forms, functions and ages.

Because the scientific community does not yet know which of the approximately 47,000 proteins are involved in various aspects of a neuron's lifespan (i.e., developing, functioning and ageing), many of the benefits of the project will be realised beyond the timescale of the current licence period. Detailing the protein composition of neurons at these stages of the lifespan will allow scientists in the field to formulate new hypotheses about how neurons form, function and age, and will be of significant value for understanding basic neurobiology, and how to address common injuries, disease or ageing of the brain.

How will you look to maximise the outputs of this work?

The applicant has a good track record in internal, national and international collaboration for research, and this will continue throughout the licence timeline. The University has a progressive policy on Open publications, providing late drafts of papers for public viewing, as well as small funds to pay fees for Open Access publication. Fundamental information (e.g., a compendium of proteins in the developing brain) are traditionally made available on a publicly available server (designated in conjunction with a funder and publisher of manuscripts) to allow broad access to the information by the research community. More direct dissemination will be pursued through peer-reviewed publication, and plenary presentations at conference events.

Species and numbers of animals expected to be used

- Rats: 69

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

Choice of Animals: The organisation of the fundamental systems in the rat brain (e.g., for moving, remembering, etc) is similar enough to our own to generate human-relevant data. In addition to this, rats are just large enough to allow for very precise dissection of small brain regions that need to be teased away from neighbouring regions so that they can be distinguished for analyses. Mice have not been chosen here, due to the fact that they are

significantly smaller in size; going beyond the limits of current dissection techniques to precisely extract the individual groups of neurons in the CNS that are most relevant for current research (particularly at early life stages).

Life Stages: The nerve cells will be studied from rats at all stages of life (from embryonic to 18 months old). This is due to the fact that the project aim is to study the proteins that are involved in the growth, functioning and ageing of these cells at all stages of life.

Typically, what will be done to an animal used in your project?

The animals will receive a non-recovery, terminal (AC) dose of anaesthesia, followed by terminal perfusion (with death confirm via Schedule 1 methods).

What are the expected impacts and/or adverse effects for the animals during your project?

This is a non-recoverable (AC), terminal procedure. The adverse effect to the animals is their placement into an anesthetising chamber.

Expected severity categories and the 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 animals will be within the non-recovery (mild) category of severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Because we do not yet know which (among the +47,000 known) proteins influence development, functioning and ageing of the CNS, we need a "real" system, with "real" cells, connected to a network of other cells order to establish fundamental, baseline information first. In essence, we cannot model ex vivo what we don't yet know about in vivo. We must establish base-line measures of neurons in their natural setting throughout the life-span. We cannot mimic or model this proteome in cell lines, because we don't yet know which proteins are being made by various groups of neurons in the normally developing and ageing nervous system.

The animals used (rats) are the least sentient mammals that have CNS connections that are similar enough to be relavant to humans. The use of rats in the project was chosen over mice due to the fact the size of the mouse nervous system is significantly smaller,

making it impossible to isolate specific tissue samples for analyses (i.e., extracting a single brain region in isolation will many times not be possible in mice, particularly in early stages of development. This would cause a confounding factor when attempting to detail the proteome of a single region)

Which non-animal alternatives did you consider for use in this project?

I have considered the use of cells lines as a replacement for (primary) animal cells:

Cells lines can be an incredibly useful replacement for animal cells when the composition of the animal's cells is known and can be designed in cell lines. General neural cell lines, for example, have been produced that allow for the expansion of neural like cells for studying general properties of these cells in culture without additional animal cells. However, no non-animal cell line exists that represent sub-phenotypes of neural cells in the brain, simply because we don't yet know what the "real" animal neural cells contain (i.e., we cannot design a non-animal cell line to mimic animal cells, because we don't yet know what they are producing) The work under this licence seeks to detail what primary cells are composed, enabling (in the future) the production of stem cell lines that mimic them.

Why were they not suitable?

Since the project's Aim is to discover the proteins that influence brain development and ageing, cell lines are not a viable alternative. In order to use cell lines, we would first need to know what proteins the cell lines should be expressing. This fundamental information can only be first gained through the use of primary 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?

Tissue will be extracted for two different types of analyses: (1) protein extraction/identification, and (2) histological analyses.

Proteomics Analyses: The estimates have been made using calculations from past research of the amount of tissue necessary to generate enough protein for mass spectrometry analyses, and for the characterisation of expression in tissue sections (e.g., histology). For small regions of the brain, 15 postnatal animals from all three life stages analysed (i.e., neonate, adult, mature adult) are necessary to generate enough material to characterise and quantify protein from the tissue (i.e., 3 x 15 = 45 postnatal animals). This estimate is based on the fact that it takes up to 15 tissue samples to reliably generate the 1-2mg of protein that is necessary to conduct mass spectrometry (proteomics) analysis. This equates to 45 post-natal animals for protein extraction of several different brain regions (as we will dissect more than one brain region per animal). Proteins expressed at

these three postnatal life stages will be compared to those expressed during embryonic development. Embryonic samples will be derived from Schedule 1 killed rats, as they cannot be perfused but (because of their much smaller size) the blood can be rinsed free of the tissue through emersion. This cannot be done with postnatal specimens as they are much too large (i.e., much of the blood will remain in tissue samples.

Histological Analyses: The estimate for the number of animals needed to characterise the expression of proteins in tissue sections (histology) across life-stages (i.e., 8 animals: 4 male, 4 female; Gomez- Galvez et al., 2020) is based on the typical number of "candidate" proteins we have generated in our past protein-based studies. Usually ~5 candidate proteins are identified for histological further histological analyses in tissue sections (Fuller et al., 2015). When conducted at the 3 postnatal life- stages analysed in the project; using 8 animals at each of the three postnatal dates, this = 24. Protein analysis within postnatal tissue will be compared with that seen in various embryonic tissue samples which will be generated by Schedule 1 methods.

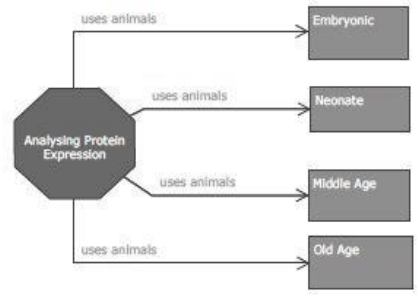
The total number of animals used in the study is 69; 45 for protein extraction of three stages of postnatal development / aging, and 24 for histological analysis.

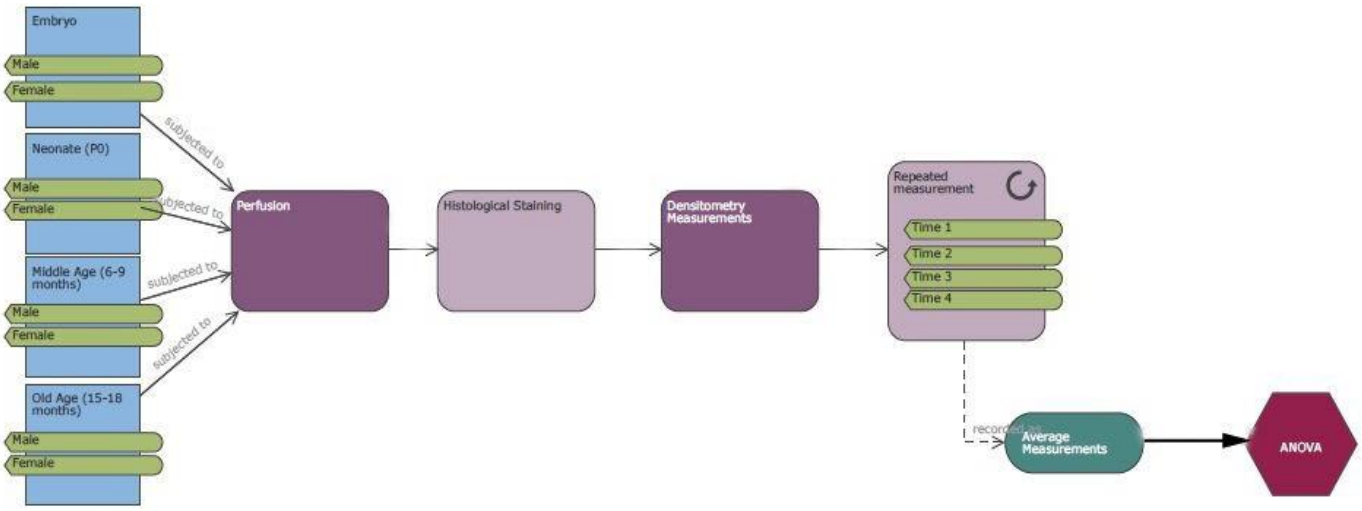
What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To calculate the number of animals needed for proteomics analysis, past data was used to quantify the number of animals needed to generate enough starting material to conduct the proteomics analyses.

In essence, the minimum number of animals needed for proteomics analysis was determined from the amount of tissue needed to reliably identify the proteins with mass spectrometry.

For histological analysis, the calculation is based on our past expression studies which have identified the number of samples needed to reliably compare densitometry measures of protein expression within tissue sections allowing. A flow chart (see below) of the histological analyses of proteins was generated using the NC3Rs EDA software (https://eda.nc3rs.org.uk/eda/landing). To assure that gender differences are accounted for, an even number of male and female rats will be used for each analysis. When using these criteria, a minimum number of 4 animals of each gender at each life stage is necessary to conduct meaningful statistical analyses. Embryonic samples used for comparison with postnatal samples will be generated through Schedule 1 methods. A histological comparison of protein expression across all life-stages will statistically analysed using a two-way ANOVA.





What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

If characterisations (i.e., histological analyses) yield highly disparate staining patterns in tissues (i.e., if there is a protein that is "present" in one and "not present" in another - where no statistical comparison is meaningful), then this will reduce the number of animals needed. However, it should be noted that this is unusual (and unlikely), and statistical differences in expression levels usually require 8 animals per group for quantifiable histological comparisons of neural 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.

The animals used will be wild-type rats. The animals used are necessary as, at present, the knowledge that is needed at a fundamental stage (i.e., the research seeks to establish the base-line for these proteomes). Because there are more than 47,000 possible proteins that cells can produce, we do not yet know how many and how much of these are made by neurons at different life stages. Pain, suffering and distress are minimised by careful handling during the euthanising. There is no lasting harm as this is a terminal procedure.

Why can't you use animals that are less sentient?

The animals chosen are the least sentient animals that are still representative to the human brain, and large enough to allow for reliable isolation of individual neurons groups. Rats have been chosen here instead of mice as it isn't presently feasible to dissect very refined regions of theCNS in the smaller animals (mice), particularly in early stages of the life-span.

Size: It is important to note that it is necessary to have enough material of a single neural group to generate meaningful information for proteomics analysis. Depending on the size of the brain structure analysed, several grams of tissue containing cells to be analysed are necessary to have enough material for characterisation work. Some areas of the brain analysed (e.g.,substantia nigra and VTA) can be very small, reaching the limits of the ability to reliably isolate these areas for analysis independent of other structures. Rats are on the cusp of having a brain large enough that some of the smallest regions could be extracted reliably (i.e., without "contaminating" the sample much with adjacent structures). The use of smaller animals, including mice, zebrafish and flies, are too small to reliable extract refined regions of the CNS in isolation for analyses.

Representative: Most animals have a brain like structure, but the organisation of fundamental areas of the brain can be substantially different from humans. The less similar the organisation of brain is to humans (e.g., such as that seen in flies, zebrafish), the more likely that the development, functioning and underlying physiology of the animal would be difficult to relate to humans. This would mean that it could be difficult to relate findings from zebrafish to humans (making the information less meaningful). Rats have been chosen here as the fundamental areas that are studied in the lab (e.g., the basal ganglia, corticospinal system, cortex, cerebellum, hippocampus) all have a very similar structural organisation to humans. These structures are important to many human maladies, such as Parkinson's disease (basal ganglia), spinal cord injury (corticospinal system), movement disorders caused by ageing (cerebellum), personality disorders (Schizophrenia, depression, bipolor disorder) and neurodegenerative diseases such as Alzheimer's (cortex and hippocampus).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedure is a terminal procedure, with careful handling of the animal as it is placed in the anesthetising chamber. The procedure is conducted in a dedicated area, with low ambient noise and light. The project licence holder will continue to liaise with the NACWO and NVS for best practice in handling animals during the procedure.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidance has been obtained from the NC3Rs website concerning both the use of anaesthesia for a wide range of procedures (https://nc3rs.org.uk/3rs-resources/anaesthesia) and for terminal procedures including exsanguination (https://nc3rs.org.uk/3rs-resources/blood-sampling/blood-sampling-mouse).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Reduction: A useful parameter to continually stay informed during the project would be sample sizing. As proteomics continues to advance, the ability to reliably characterise the protein content of a sample of brain tissue can become less and less. If the field advances significantly in this way, it could be possible that fewer animals would be needed to obtain the material necessary to do the analysis (effectively providing the opportunity for Reduction of the number of animals needed). The lab publishes in peer-reviewed journals on new methodologies for protein analysis (Orme et al., 2010), and will continue to review such publications to determine if such advances evolve during the project timeframe.

Refinement or Replacement: The applicant will maintain a commitment to consulting 3R's publications to monitor updates and developments on the guidelines. Further to this, the applicant will continue to keep abreast of progress in the fields of neuroscience and cell biology with frequent literature searches to ensure that if suitable alternatives or more refined methods become available, then then they will be adopt quickly. Finally, maintaining currency in research literature, reviewing funding applications for Councils and charities, and direct interactions with researchers within the field will ensure that the work is not being duplicated or superseded elsewhere.

73. Mechanisms of Aortic Stenosis

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 valves, Aortic stenosis, Calcification, Tissue engineering, Cardiovascular

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant
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 project aims to understand the mechanism of calcification in cardiovascular disease and develop a potential treatment for the disease.

To assess pharmacological strategies that may be used to provide a rationale for the medical treatment of aortic stenosis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Valve calcification remains a major medical problem that can only be resolved by replacing the valve. The development of a medical treatment for this disease relies on understanding

the cellular and molecular events that lead to the calcification process. Therefore, it is crucial to further understand the mechanisms to identify potential therapeutic targets that could slow down or reverse calcification without necessitating invasive surgical procedures. This disease affects a significant portion of the population, particularly the elderly, leading to conditions such as aortic stenosis, which can drastically impact the quality of life and survival rates. Current treatment modalities are limited to surgical interventions, which carry their risks and complications, especially in patients with multiple comorbidities.

In-depth research into valve calcification could unravel the complex interplay of genetic, environmental, and metabolic factors that contribute to this pathological process. For instance, understanding the role of lipid metabolism, inflammation, and oxidative stress in calcification could lead to the development of drugs targeting these pathways. Research could also identify biomarkers for early detection, allowing for treatment initiation before the disease progresses to a severe stage. Furthermore, insights from valve calcification studies could inform our understanding of other calcific diseases, such as vascular calcification, contributing to a comprehensive approach to managing these conditions.

The potential for a medical treatment that could delay or prevent the need for valve replacement surgery would not only improve patient outcomes but also significantly reduce healthcare costs. Ultimately, the knowledge acquired could drive innovations in biomedical engineering, such as developing bioprosthetic or tissue-engineered valves that more closely emulate the natural valves' biological and mechanical properties. In summary, researching the pathophysiology of valve calcification is critical for advancing medical treatments, improving patient care, and potentially offering ground-breaking insights that could affect a wide range of calcification-related health issues. It is a vital step towards enhancing the life expectancy and quality of life for patients suffering from this debilitating condition.

What outputs do you think you will see at the end of this project?

Our goal is to gain a deeper understanding of the mechanisms involved in aortic valve disease through our studies and to identify potential molecular and pharmacological targets that can be used for treating the disease. The data collected from these studies will help us better understand the disease process and provide a foundation for future research.

Findings will be made available to other scientists through publication in peer-reviewed journals and presentations at scientific conferences and meetings. The genetically altered animals developed may also be valuable to other scientists.

Who or what will benefit from these outputs, and how?

These studies aim to provide comprehensive insights into the mechanisms underlying aortic valve disease. By evaluating the effects on mice that manifest aortic stenosis, whether through breeding with animals with targeted gene deletions or via treatment with pharmacological inhibitors, we intend to pinpoint molecular and pharmacological targets. Such targets are crucial for understanding the disease and disseminating findings through scientific publications in the short term. In the long term, the testing of new therapeutic agents on these animal models, extending potentially to larger species, will deepen our grasp of the disease's intricacies.

This progression of research is designed to catalyse the development of clinical trials, fostering novel medical treatments for aortic stenosis. A critical goal of these endeavours is to reduce the reliance on surgical valve replacement, which is often fraught with risks and leads to significant patient morbidity. By identifying methods to delay or obviate the necessity for such invasive procedures, we can alleviate the strain on healthcare resources and enhance patient experiences by mitigating the stress and risks associated with major surgeries.

Furthermore, these investigations are pivotal for the advancement of tissue-engineered heart valves. The potential to cultivate a valve that not only mimics the biological and mechanical functions of a natural valve but also integrates seamlessly with the patient's own tissues could revolutionise the standard of care. The benefits of tissue-engineered valves are multifold: they promise a reduction in the incidence of rejection, diminish the requirement for lifelong immunosuppression, and offer the prospect of valves that grow with paediatric patients, thereby minimizing the need for multiple surgeries over a lifetime. Such innovations, by improving the compatibility and longevity of valve replacements, could significantly enhance the quality of life for patients.

How will you look to maximise the outputs of this work?

We plan to publish our research findings in open-access journals, present at scientific meetings, and share resources such as data, animals, and tissues with other researchers.

In addition to publication, we will collaborate with academic and/or industrial partners to develop potential therapies and treatments for valvular disease.

Species and numbers of animals expected to be used.

- Mice: 702
- Rats: 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.

The B6;129S-Ldlr<tm1Her>Apob<tm2Sgy>/J mouse line is genetically predisposed to atherosclerosis, rendering it an effective model for investigating the disease's progression as seen in humans. In our study, we utilise adult mice because they exhibit arterial narrowing due to calcification, a condition that can be reliably replicated through dietary modifications and drug administration via their drinking water.

For our experiments, we have opted for adult wild-type rats rather than younger ones. This choice is guided by the need for optimal adhesion and to minimise the risk of stretching the implanted patches. Young animals are still growing, and their changing body dimensions could potentially disrupt the adhesion process of the patches. By using adult rats, whose

growth has stabilised, we ensure a more consistent and controlled experimental environment.

Typically, what will be done to an animal used in your project?

Animals in our study will experience dietary changes, which, while not anticipated to cause distress, may occasionally lead to obesity or itchy skin. Certain diets might also cause weight loss due to unpalatability. If an animal loses 10% of its body weight, it will return to a normal diet. In addition, we will administer drugs through drinking water for a period of up to six months.

For the study of polycaprolactone (PCL) scaffold responses, two surgical procedures are planned:

Subdermal Pouch Surgery: This procedure, lasting 30 minutes to an hour, involves creating a small dorsolateral incision in the skin to form a subdermal pouch. We will then insert the PCL scaffold material into the pouch, ensuring it lies flat. The insertion site will be flattened to re-oppose the wound edges, after which the skin will be sutured or stapled back together. This process will be repeated on the opposite side of the backbone to create a second pouch. Following surgery, the rat will be placed in a recovery cage for full recovery.

Aortic Patch Surgery: Lasting between 30 minutes and 1.5 hours, this surgery involves a midline abdominal incision to access the abdominal aorta between the renal artery and the inferior mesenteric artery. The aortic branches in this segment will be ligated, and the arteriotomy will be closed with the scaffold material using fine sutures. We will then ensure haemostasis and restore blood flow to the aorta and surrounding vessels. The skin incision will be closed, and the perfusion of blood to the hind limbs assessed via pulse checks and temperature measurements using a probe. The rat will then be placed in a recovery cage to recover fully.

Post-surgery, animals are expected to recover rapidly from anaesthesia, typically within two hours. They may experience some discomfort and mild to moderate pain post-operatively, which will be managed with appropriate analgesics.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals will have minor surgery to implant scaffold material under the skin. They are expected to recover quickly and will be given painkillers and post-operative care just like people recovering in a hospital.

In the case of wound dehiscence, wounds may be re-sutured or re-stapled on one occasion within 48 hours of the initial surgery only if the incision is clean and shows no discharge.

PCL Scaffold material that contains pharmacological agents could be released into the circulation, causing systemic effects in rats. however, these effect should be minimal, as the device is design as a local control drug release device.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Breeding and maintenance of GA Mice, 100% mild. The mice show no phenotypical changes compared to wild-type mice.

The severity of Surgical procedures on rats would initially be moderate (100%), During recovery together with analgesia it would be reduced to mild (100%).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In vitro work on valve cells, aimed at determining the influence of the mediators we intend to study, has either been conducted by our laboratory or is well-documented in the literature. However, to fully understand the mechanisms behind the processes involved in aortic stenosis, it is important to have an intact functional heart and full blood circulation to fully comprehend the mechanisms involved in aortic stenosis. This allows for the cells to be exposed to the physiological conditions of flow and pressure, along with circulating and locally released mediators, which are all crucial for understanding the processes involved.

Which non-animal alternatives did you consider for use in this project?

As a part of our project, we conducted a thorough evaluation of potential non-animal alternatives, which involved a critical analysis of in vitro cell culture systems and microfluidic devices.

Why were they not suitable?

It is not possible to accurately replicate the physiological conditions of flow and pressure in the presence of circulating and locally released mediators in vitro and maintain tissue viability over a prolonged period. Such an in vitro system also lacks blood components and does not replicate in the complexity of a multicellular system. We therefore wish to assess the loss or gain of function of specific mediators on the development of aortic stenosis in the whole animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In addition to literature search data and analysis of tissues from previous pilot studies, this will give us information on how to determine the variability and minimise the group size. This will ensure maximum efficiency of animal use.

Using typical variations from our own earlier experimentation to calculate the minimum number of animals to be used whilst ensuring that the results are statistically significant. Sample sizes for our experiments are estimated from past experiments. Calculations typically show that we need group sizes of 8 to achieve the quality of results we need. The number of animals we will require for breeding has been estimated using the data from our annual procedure returns.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will avoid unnecessary production or import of genetically altered animals by searching both internal and external databases and cryobanks (e.g. NC3R’s mouse database, Mouse Locator, Cre transgenic database). The individual models we intend to use have already been fully characterised and require no further development. Breeding strategies are performed by qualified personnel and are aimed at avoiding unnecessary animal generation (animal surplus). Homozygous mouse breeding be used, as both genders are viable and fertile.

After the results of our feasibility studies, we will follow the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental design, practical steps, and statistical analysis. The EDA provided 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 assist animal users.

Statistics:

For statistical considerations, we will consult with the university's statistical advisory service, which will provide advice on group size, and biological and experimental repeats. Data will be published according to the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have also explored alternative methods and approaches to reduce the use of animals in our experiments. For example, where possible, we use ex vivo material from tissue banks or surplus stock.

After the experiments, we will collect as many tissues as possible during the post-mortem process. If we do not need to analyse the tissues immediately, we will freeze them and make them available to other researchers studying similar topics.

Colony maintenance of genetically modified and mutant animals will be performed with homozygous breeding pairs whenever both genders are viable and fertile, and colony sizes will be kept to a minimum. Cryopreservation of gametes and embryos to archive lines will avoid unnecessary continuous breeding.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Currently, only mice are accessible to the complex genetic manipulations required for the development of aortic stenosis. Changes in diet and the administration of drugs are the only interventions required for this project; no surgery is needed for these models to develop aortic stenosis. The transgenic mouse models are commercially available, and their disease progression has been thoroughly documented.

For the surgical PCL patch experiments, wild-type rat strains are used. Improvements have been made to enhance the care and well-being of animals after surgery. These include improved monitoring with welfare score sheets, post-operative care, and pain management regimes.

Why can’t you use animals that are less sentient?

To recapitulate and develop the stenosis disease stage, adult mice are required.

Rats in the adult age range possess the appropriate physiology to understand the effects of calcification on implanted materials, which is relevant for PCL implant investigations.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Minimising animal suffering will be achieved by ensuring the technical skills and competence of all personnel involved in animal care and use. Improvements have been made to enhance the care and well-being of animals after surgery. These include improved monitoring with welfare scoresheets, post- operative care, and pain management. Humane endpoints will be established as suggested by Morton in his 2000 work, 'A Systematic Approach for Establishing Humane Endpoints.

Clinical/behavioural signs (such as posture and activity), and pathophysiological parameters (such as respiration rate and weight loss) will be used with the Mouse Grimace Scale (MGS): together with the Facility scoresheet to determine humane endpoints.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Morton in his 2000 work, 'A Systematic Approach for Establishing Humane Endpoints.'
NC3Rs' experimental design guidance and experimental design assistant.
PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have subscribed to the NC3Rs newsletter and will regularly check their website for updates. Regular discussion with colleagues to discuss the latest NC3Rs advancement and potential application to the experiments within the parameters of the project licence.

74. Receptor signalling in the intestine

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Hormones, Nutrient-receptors, Intestinal ion transport, Intestinal motility and transit, Obesity, diabetes and colitis

Animal types	Life stages
Mice	juvenile, adult, embryo, neonate, pregnant
Rats	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to establish the protective effects of endogenous hormones and neurotransmitters found within the healthy gut. Also, we will investigate the receptor mechanisms that are protective, resulting in anti-obesity, anti-diabetic and anti-inflammatory activities derived from the gastrointestinal (GI) tract.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The rates of obesity and diabetes are escalating world-wide and the NHS costs for treating these preventable diseases are likely to increase by £2 billion/year by 2030. Certain types

of gastric bypass surgery reduce body weight significantly long-term and can also reverse diabetes in man, but we do not understand how re-plumbing the gut leads to this apparent cure. Gut hormones and their receptors are thought to play a part. This work will improve our understanding of how hormones produced by cells in the gut lining, modulate gut functions. The digestion of nutrients and their transit and absorption vary along the gut length and change during gut inflammation (colitis). It is becoming clear that the signalling between key cell types (e.g. neurons and hormone-containing epithelial cells) is necessary to translate nutrient stimuli in to appropriate whole-body responses, but there are significant gaps in our knowledge. This project will resolve some of the multi-cellular, gut-specific mechanisms and, provide a basis for better understanding how changes in gut functions can contribute to diseases such as obesity, diabetes and colitis.

What outputs do you think you will see at the end of this project?

The project will provide new scientific information crucial ultimately for the development of novel, improved anti-inflammatory, as well as anti-obesity and anti-diabetic therapies. Understanding the mechanisms that underpin intestinal function in health and disease e.g. upon stress-induced changes or mucosal erosion during inflammatory bowel disease (IBD), is fundamental for improvements in therapeutic strategies to treat bowel dysfunction. In time, this novel data will contribute potential in harnessing the capacity of the intestine's epithelial lining to renew itself and/or secrete anti- inflammatory mediators to minimise mucosal erosion; or to enhance intestinal hormones to cause satiety and over a longer time-course then tackle obesity and diabetes, and in-so-doing avoid the risks of gastric bypass surgery.

By assessing the protective effects of intestinal mechanisms involved for example, in GPR35-specific pathways (using novel selective GPR35 ligands) in healthy intestine, these discoveries will inform investigations of inflamed intestine, and establish the receptor's protective potential in models of IBD. Our research will also provide insights into intestinal side effects i.e. constipation or diarrhoea of new receptor-targeted therapies. We will publish this research in open access journals and present our findings at scientific meetings.

Who or what will benefit from these outputs, and how?

In the short-term, beneficiaries will be our own and our collaborators' groups, plus GI researchers at national and international levels. In the medium-term, scientists in the pharmaceutical and clinical groupings will benefit from reading the papers we publish in scientific journals and online open access archives. For research funded by non-charitable agencies where the identity of particular novel compounds may be restricted, we optimise the opportunities to publish our research by designing experiments that compare novel drugs in parallel with commercially available ligands with similar pharmacology. The hope is that long-term, patient groups will benefit from novel therapies with increased efficacy and fewer side effects.

How will you look to maximise the outputs of this work?

We will continue to collaborate with academic and pharmaceutical partners with the aim of publishing our data in open access journals and databases. We share our data and knowledge regularly with collaborators in academia and pharmaceutical companies e.g.

with colleagues at Imperial College and Heptares Therapeutics, via twice-monthly meetings. This efficient method of dissemination will continue.

Species and numbers of animals expected to be used

- Mice: 5,000
- Rats: 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 will use young adult male and female mice or rats, 10-20 weeks of age. These animals, including naturally-occurring genetically-altered rodents, are models of diabetes and obesity, or colitis, and will be used accordingly. The structure of the mammalian gut and its intrinsic nervous system are complex and hormones such as peptides or 5-hydroxytryptamine (5HT) are found in specialised (endocrine) cells in the inner lining of the gut, but these cells are rare. Notably, these types of enteroendocrine cells (EECs) are similar in mice, rats and humans, and the hormone combinations that are released from different endocrine cell types are similar, sometimes the same, e.g 5HT activates 5HT3 and 5HT4 receptors in gut mucosa of all three species. Thus rats or mice are appropriate models in this context.

Additionally, the receptors that are activated by certain peptide hormones e.g. peptide YY (PYY) are the same in mouse and human gut (Y1, Y2 and Y4 receptors), and so here the mouse gut is the most appropriate model for human GI mucosa.

Rats are also included because certain naturally-occurring genetically-altered strains e.g. Zucker diabetic fatty rats, are an accepted model of obesity and type 2 diabetes.

Typically, what will be done to an animal used in your project?

Animals will be bred to produce GA mice.

Animals may be fed an altered diet e.g. high in fat, typically for between 2 - 10 weeks, after which they will be killed humanely and GI and other tissues used for *in vitro* analyses.

Some animals will undergo restraint or novel environment stress over 3-5 consecutive days.

Colitis experiments will typically last for two weeks. Animals will drink a dextran (1% DSS in tap water) ad lib for typically 6 days, after which 2 days of tap water will allow some recovery, and administration by either oral gavage of i.p. drug or vehicle, followed by a further 6 days drinking DSS. Animals will then be killed humanely and tissues used for *in vitro* analyses.

What are the expected impacts and/or adverse effects for the animals during your project?

Adverse effects are not expected with the genetically-altered animals we propose to use. They breed and behave normally and are robust and healthy. Some naturally-occurring genetically-altered strains will be obese/diabetic (e.g. ZDF rats), and these animals will be over-weight and diabetic with glucosuria in adulthood.

No adverse effects have been observed after colonic administration of a glass bead used to measure colonic transit, but observation of animals will occur daily looking for signs of diarrhoea or constipation, prolapse or abnormal movement, or loss of condition. Animals exhibiting any unexpected harmful phenotypes exceeding mild severity will be killed humanely, or in the case of individual animals of particular interest, advise will be sought from the local HO inspector.

Restraint or novel environment stress, will induce mild, transient diarrhoea and observation of animals looking for signs of chronic diarrhoea, will occur daily. Animals exhibiting any unexpected harmful phenotypes exceeding mild severity will be killed humanely, or in the case of individual animals of particular interest, advise will be sought from the local HO inspector.

The colitis models will cause colonic inflammation with consequent diarrhoea and possible rectal bleeding, weight loss, abnormal movement and loss of condition. These adverse effects will be minimised by using a short, mild inflammatory stimulus for example, delivering a dextran (DSS at 1%) in drinking water for typically up to 6 days, checking animal health and condition daily. If an animal shows an abnormal condition/behaviour, then the advise of the NACWO and/or NVS will be sought and if the condition remains for 24h the animal will be killed by a Schedule 1 method. At the end of the experiments the animals will be killed humanely and tissues collected for analysis.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Genetically-altered animals are expected to continue to be robust and healthy (sub-threshold). For naturally-occurring genetically altered rats e.g. ZDF rats, all of these animals develop obesity and T2D as adults. All animals fed a high fat diet (HFD) will gain weight over the period of altered diet. Approximately 50% of mice fed a HFD are expected to become diabetic within 6 weeks.

80% of animals that undergo restraint or novel environment stress are expected to exhibit mild, transient diarrhoea.

The DSS colitis model is expected to cause mild to moderate colonic inflammation. Approximately 50% of mice or rats drinking 1% DSS for typically 6 days, lose 1-5% of body weight, and passage of soft consistency stools but that are still formed (i.e. a disease activity index, DAI of 1.0). A second cycle of 6 days of 1% DSS after a 2 day break drinking tap water, results in animals losing 2-5% of body weight, and 75% of animals exhibit mild diarrhoea (soft stools) with positive hemocult (DAI of 1.0-2.0). At the end of the

experiments the mouse or rat DAI is recorded, and they will be killed humanely, and tissues collected for *in vitro* analyses.

Overall, 50% of mice, and 90% of rats will experience mild severities. <5-10% of animals are expected to experience moderate severities.

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 anatomy of the gut wall is complex. While it is possible to maintain mucosal epithelia and hormone-containing endocrine cells within this layer in culture for short periods of time, there is no non- sentient system that models the mammalian GI tract with its enteric nerves or extrinsic nerves from the peripheral nervous system, or that can replace the use of animals.

We have performed co-culture of myenteric nerve explants with epithelia but the epithelial lining is innervated predominantly by the inner submucosal plexus neurons and these are;

smaller in size and much more difficult to isolate and maintain in culture, and are,

lower in number, so their depolarisation provides for smaller, transient signals that are consequently much more difficult to characterise.

Organoids derived from human or rodent intestine have been grown and developed by other groups, however these alternatives do not include enteric nerves.

We have instead used in vitro assays of native rodent GI mucosae where importantly enteroendocrine cells and submucosal enteric nerves remain intact, innervating the mucosa across which we measure epithelial function electrophysiologically and we compare these models with human colon mucosa. We can also measure smooth muscle contractility in isolated preparations from rodent intestine and human colon. There are notable significant similarities in peptide-receptor mechanisms within the mucosa of mouse and human colon but the availability of healthy human tissue is limited. Comparison of outcomes from mouse, rat and human specimens will continue to be used wherever appropriate and will compliment the data from whole animal GI transit experiments.

Which non-animal alternatives did you consider for use in this project?

Adenocarcinoma cell lines derived from colon tumours can be grown and maintained in culture to form polarised epithelial layers. We have characterised several human cell lines over the last 20 years.

We have also co-cultured myenteric nerve explants with tumour-derived colonic epithelia. Studies with submucosa plexus explants have not been performed because *in vitro* study of native intestinal tissues is the gold standard.

Organoids derived from human or rodent intestine have been grown and developed by other groups, however these alternatives do not include enteric nerves. Our initial collaborations with experts in animal intestinal organoid culture and patient-derived GI organoids and scaffolds (e.g. https://doi.org/10.1038/s41596-022-00751-1) will inform our research through joint group meetings.

Why were they not suitable?

All the colonic cell lines we have characterised to date lack any enteric innervation as well as inflammatory cells, and they also lack some key receptors for hormones and/or neurotransmitters that are present in native intestinal and colonic mucosae.

In terms of co-culturing epithelia with enteric neurons; the native epithelial lining is innervated predominantly by a different set of enteric neurons, i.e. the inner submucosal plexus nerves and these are:

smaller in size and much more difficult to isolate and maintain in culture, and are;

lower in plexi number, so their depolarisation provides for smaller, transient signals that are consequently more difficult to measure and to characterise.

Intestinal organoids are expensive to grow and maintain long term in culture, requiring significant funding for consumables. More importantly, mature organoids do not include enteric nerves and thus have limited utility for studying neuro-epithelial 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?

Extensive previous experience measuring GI transit *in vivo* indicates that the numbers of animals required for statistical significance to be revealed is n = 8-10 (Tough *et al*. 2011; Forbes *et al*. 2012; Moodaley *et al*. 2017; Tough *et al*. 2018). The estimated number of animals is based on planned experiments to be performed by funded full-time staff working under licence.

When novel compounds are administered, pilot studies will utilise 2-3 animals initially per group.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Where appropriate we perform *in vitro* experiments with intestine from specific GI regions first, in order to address the basic mechanistic questions prior to application in whole animal studies. We will also utilise the NC3Rs EDA wherever possible.

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 of GA animals will be followed.

Initially when testing a novel ligand, we perform *in vitro* studies first, e.g. we measure natural faecal pellet excretion using isolated colonic specimens prior to performing any *in vivo* colonic bead transit studies. This approach has over the last 8-10 years, been shown to accurately predict the modulatory effects of a peptide or amine specific agonists or antagonists (Forbes *et al*. 2014; Moodaley *et al*.
2017; Tough *et al*. 2020).

We regularly share tissues from the same animal so that for example, *in vitro* studies of specimens from different GI regions can be investigated in parallel.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically-altered animals e.g PYY-/-, are expected to continue to be robust and healthy (sub- threshold). For naturally-occurring genetically-altered rats e.g. ZDF rats, all of these animals develop obesity and T2D as adults. We have extensive experience with germline-modified mice and comparison of the phenotypes in these models will allow us to refine (and reduce) animal numbers.

Mice and rats will also be used to provide isolated GI tissues for in vitro mechanistic investigations initially. These will inform subsequent in vivo studies.

Some animals will be fed an altered diet e.g. high in fat, typically for 1-6 weeks, to induce alterations in intestinal physiology and result in obesity and diabetes. The colonic transit rates will be measured by glass bead insertion, measuring the time to bead excretion and comparing this with natural faecal pellet excretion, which will permit refinement and reduction of animal numbers.

Charcoal meal delivery by gavage is a proven method for measuring upper GI transit, typically over a period of 30 min. Animals are conditioned to handling prior to gavage in order to minimise stress. All transit rates will continue to be compared with the rates obtained in previous studies performed by my group over the last decade.

During the out-going licence we optimised a mild colitis model using the dextran, DSS. This mouse model exhibits some areas of focal inflammation, small reductions in body weight and colonic length, but no apparent change in upper GI or colonic transit rates. Our pathologist collaborators have confirmed that focal mucosal inflammation occurs after one and two periods of 6 days drinking DSS (at 1% in drinking water, with 2 days drinking tap water in between DSS dosing periods). We will adopt this refinement in future colitis studies when testing novel compounds that (also based on in vitro studies) are predicted to protect the gut mucosa from inflammation and mucosal erosion. Acute stress, such as restraint stress will be included initially for some animals that undergo mild colitis in order to establish whether this stressor worsens DSS-induced colitis, before chronic stress is included. This incremental approach will inform our understanding and, using chosen drug treatments, will allow us to interrogate receptors involved in modulating motility pathways at an early phase of GI dysfunction.

Why can't you use animals that are less sentient?

Our research shows that the GI peptides (peptide YY (PYY), neuropeptide Y and pancreatic polypeptide) and amines (5-HT, for example) and their receptors show that the mouse GI tract is the best rodent model pharmacologically for the human intestine. The same trio of Y receptors are expressed in the same mucosal cell types, and these receptors are stimulated by the same peptides in both species. In disease models, such as the naturally occurring genetically-altered ZDF rats that develop obesity and diabetes, we have established that GI nutrient-sensing mechanisms initiated by fats that are GPR119 agonists, cause acute PYY and GLP-1 release, as observed in isolated human GI mucosa. This strategy continues to inform our mechanistic understanding of GI mucosal chemosensory pathways and the epithelial sidedness of this complex mucosal signalling (Tough *et al*. 2018; Tough *et al*. 2020).

Non-mammalian models do not replicate the complexity and diversity of hormones and their receptors within the GI tract.

Insufficient information on the development of signalling pathways in younger animals has been published to date, thus we may need to use tissue from younger animals in future studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Adverse effects are not expected with the genetically-altered animals we propose to use. They breed and behave normally.

No adverse effects have been observed after insertion of a glass bead to measure colonic transit, but observation of animals will occur daily looking for signs of diarrhoea or constipation, prolapse or abnormal movement, or loss of condition. Animals exhibiting any unexpected harmful phenotypes exceeding mild severity will be killed humanely, or in the case of individual animals of particular interest, advise will be sought from the local HO inspector.

The colitis models will cause colonic inflammation with consequent diarrhoea and possible rectal bleeding, weight loss, abnormal movement and loss of condition. These adverse

effects will be minimised by using a short, mild inflammatory stimulus for example, delivering a dextran (DSS at 1%) in drinking water for typically up to 6 days, checking animal health and condition daily. If an animal shows an abnormal condition/behaviour, then the advise of the NACWO and/or NVS will be sought and if the condition remains for 24h the animal will be killed by a Schedule 1 method. At the end of the experiments the animals will be killed humanely and tissues collected for analysis.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our optimised mouse model of mild colitis was influenced by the work published by Wirtz et al. (2017; doi:10.1038/nprot.2017.044) for example, who showed that 1-2 weeks of DSS administration generated an improved mouse model of inflammatory bowel disease (IBD) that better reflected the mucosal cellular nature of IBD in human GI mucosa. Subsequently, our own studies provided GI specimens for our pathologist collaborators who confirmed that focal mucosal inflammation results from both one and two periods of 6 days of DSS dosage (at 1% in drinking water, see above). We will use this refinement in future studies when testing novel compounds that (based on *in vitro* studies) are predicted to protect the gut mucosa from erosion.

We will follow the guidance for ‘Refining procedures for the Administration of substances’ (https://doi.org/10.1258/0023677011911345) and for aseptic surgical procedures (at: https://www.lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf)

We will also follow the recommendations laid out in the Prepare Guidelines checklist.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Reading the scientific literature in open access journals. For example, our initial collaborations with experts in animal GI organoid culture and patient-derived GI organoids and scaffolds (e.g. Meran L. *et al*. 2023; https://doi.org/10.1038/s41596-022-00751-1) will inform our research through regular group and departmental discussions, as well as discussions with scientific colleagues at national and international conferences.

We will continue to read NC3Rs newsletters and articles, including checking 3Rs-related websites regularly.

75. The social behaviour circuitry of zebrafish in health and disease _Version2

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Social behaviour, Zebrafish, Development, Somatosensory system, Social network

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our brains are innately predisposed towards sociability. The harmful effects of loneliness and social isolation on our mental and physical health have become more apparent in the aftermath of the COVID-19 pandemic. However, the exact impact of reduced or non-existent social interactions on various aspects of our bodies remains largely unknown.

The objective of this project is to characterise the underlying circuits responsible for social behaviour in zebrafish during their development, thereby enhancing our understanding of how these circuits might be affected in different scenarios such as social isolation, mental disorders, and genetic alterations.

Moreover, we seek to understand how this social circuit is modulated by other prominent stimuli, such as somatosensory and nociceptive stimuli (e.g., touch, pressure, vibration, heat, etc.). Previous research has indicated that these stimuli can influence social behaviour, and our aim is to identify the specific brain regions involved in this modulation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could

be short-term benefits within 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 utilizing zebrafish as an animal model to examine social behaviour and preferences in juveniles, it will be possible to:

Investigate how the social preference circuit is established during early development in an animal model that develops ex utero and maintains transparency during the initial weeks of development.

Determine the early environmental factors that contribute to impairments in the anatomy and function of the social circuit, and characterize the underlying mechanisms.

Create zebrafish mutants for genes identified as mutated in humans with social behaviour disorders. This will facilitate the development of new zebrafish models (e.g., for schizophrenia, bipolar disorder, etc.) and help characterize the role of these specific genes.

Understand how social behaviour can be influenced by other prominent sensory stimuli (e.g., visual, acoustic, somatosensory stimulations) that have the potential to induce behavioural changes, and modulate the threshold of these responses.

What outputs do you think you will see at the end of this project?

By the conclusion of this project, we aim to have an improved understanding of the areas, neurons, genes, and neurotransmitters that constitute the social brain network and how they process social information. We also aim to identify key genes involved in the pathophysiology of diseases that impair social behaviour, characterize the effect of environmental factors such as social deprivation, and understand how the social circuit can be modulated by other sensory circuits.

We anticipate that this project will result in 3 to 4 major publications within the next 5 years.

As we will also be investigating the impact of small molecules on the modulation and rescue of social behaviour, and characterizing specific genetic mutations found in individuals with social behaviour disorders (e.g., schizophrenia, bipolar disorders, etc.), there may be potential to identify targets for new therapeutic approaches.

Who or what will benefit from these outputs, and how?

The overarching goal of this research project is to characterize the development of the social preference network in zebrafish, both in health and disease states. Completing this work will further establish zebrafish as a valuable animal model for studying human diseases that exhibit impairments in social behaviour, and could potentially reveal new therapeutic targets.

In the short term, this project will aid other researchers in understanding the visual features that social animals use to recognize their peers, the implications of impairments in these

circuits during development, the role of early social visual experience in establishing social preference, and the composition of the social rewards network in zebrafish.

By studying the complete development of the social circuit in a living animal, from birth to maturation, we aim to answer several questions in developmental neuroscience. For example, we will be able to determine whether certain social brain areas develop at different stages, or if the interconnections between social regions play a key role in functional specialisation. Furthermore, we will examine whether the social network undergoes a process of refinement and maturation based on synaptic pruning (the process of synapse elimination that occurs between early childhood and the onset of puberty in humans), and how experience and early environmental intervention influence later stages of development.

This project will also clarify the visual stimuli that are sufficient and necessary to drive social preference. For instance, by characterizing the neurons that process different social visual features (e.g., stripes vs dot skin patterns) both anatomically and functionally, we will contribute to the understanding of how specific features of social stimuli are processed by different neurons.

In this research, we also plan to study the effects of social isolation and other environmental factors (e.g., rearing fish with conspecifics with different skin patterns over time) on social behaviour. These experiments will be very useful for animal welfare and research. For example, we have already shown that early isolation affects sociality later in life, and we have identified the key areas involved.

Utilizing our well-established social preference test, we will continue to generate and analyse zebrafish with mutations for genes that have been linked to individuals with social disorders (e.g., ASD, Rett Syndrome, Fragile X Syndrome, schizophrenia, etc.). By studying these genes, we hope to gain insights into their role in the pathophysiology of the disease and, potentially, identify new targets for clinical treatment.

Finally, we will use the behavioural tests we developed to study how various sensory, somatosensory, and subthreshold noxious stimuli (stimuli that do not cause tissue damage, but are capable of activating noxious receptors) can modulate social preference, and vice versa. For instance, it is well known that positive social interactions can reduce the threshold for pain in humnas, however, the underlying mechanism remains unknown.

In summary, the data collected in this project will provide crucial information for the care and husbandry of zebrafish, contribute to basic research, and have potential applications in translational medicine.

How will you look to maximise the outputs of this work?

We plan to take several steps to ensure our research and findings are disseminated widely:

Manuscript Publication: We have an excellent track record of sharing our findings with the academic community through publications in peer-reviewed, open-access journals that reach a wide scientific audience.

Meetings and Seminars: We are connected to an extensive network of first-class international researchers worldwide, and we successfully share resources, expertise, and conduct weekly lab meetings.

European and International Conferences: We will further promote our research to scientists in the same and different fields by attending and presenting at significant conferences.

Online Dissemination: We will keep our website updated with the latest research findings, manuscript summaries, protocols, transgenic and mutant lines, raw data, custom software, etc. Our website will facilitate immediate knowledge and resource dissemination. A testament to our commitment to sharing findings before publication is our practice of uploading our papers to bioRxiv, a free archive and distribution service, prior to publication. We will also write press releases and research highlights and use social media platforms, such as Twitter and Facebook, to communicate results.

Engagement with Charities: During our research, we will initiate communication with charities working with families affected by various diseases (such as autism and schizophrenia). Our aim is to keep them informed about our progress in understanding social behaviour and how social interactions may make pain more tolerable.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 698,000

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 research project aims to characterize the social preference circuit in zebrafish, both in health and disease states. Given the complexity of social preference as a behaviour, it necessitates observing how the whole brain processes visual and sensory social information in a living animal.

Although various non-protected animals exhibit different levels of social behaviour, zebrafish emerge as the best choice as an animal model for this project for several reasons:

Thanks to their optical transparency during embryonic and larval stages, they offer a viable alternative to mammalian models. Many observations can be conducted in a far less invasive way. For example, in vivo imaging of neural activity can be performed non-invasively in either free-swimming or tethered larvae where the entire brain can be imaged with single-cell resolution. These experiments allow for the monitoring of hundreds of cells simultaneously, which in turn reduces the number of experiments needed.

They are the least complex vertebrate model exhibiting complex social behaviours. Our lab was the first to develop a test to study social preference in juvenile zebrafish (10-16 mm total body length), as opposed to adult fish.

Their development occurs *ex utero*, allowing us not only to track the development of the entire social preference circuit from fertilisation, but also to readily manipulate the circuit or test the effects of environmental and genetic factors.

In conclusion, there is currently no cell, computer model, or equivalent non-animal system that can accurately and efficiently model these phenomena.

Typically, what will be done to an animal used in your project?

The research proposal aims to characterise the development of the social preference and its underlying circuit. Therefore, the project involves the use of different techniques from anatomical to functional and behavioural. For instance:

We will use either wild/type, GA, or fish that have undergone a genetic or environmental manipulation (e.g. social deprivation) or have been treated with small compounds that alter sociality.

These fish, or their offspring, will be screened for changes in a multitude of different behaviours by using custom-built tests developed in the lab. For example, we will use free-swimming social test to identify changes in sociality, somatosensory experiments to test for changes in tolerance to sensory stimuli, visual experiments to test for changes in visual acuity or eye gaze, etc.). The behaviour of the fish will be recorded with cameras to identify changes in their swimming patterns.

At the end of the experiment, the brain of some of these fish will be used to identify changes in functional activities to identify key brain areas by using several methods such as immunohistochemical experiments, which allow labelling of specific RNA or proteins, and high-resolution microscopes. These experiments will allow to identify key areas without having to use additional fish for functional imaging.

Only once we will have a good understanding of the social areas involved, we will start to perform targeted imaging experiments to look at neuronal activity and responses to different stimuli in living animals. These fish will be embedded in an agarose gel (a matrix that allows to restrain without harming) to be able to observe brain activity with high resolution.

Some of the fish will be exposed to small compounds to test specific conditions (e.g. identify what pathways are involved in social behaviour processing, identify compounds that rescue of sociality, etc.)

In some experiments we will also assess changes of activity and sleep behaviour in free swimming fish. Fish will be simply tracked with video cameras while they sleep over a few days.

What are the expected impacts and/or adverse effects for the animals during your project?

This research project aims to study changes in social behaviour under various conditions and treatments.

Most experiments will involve tracking larval or juvenile zebrafish in different arenas alongside their siblings to identify changes in sociality, locomotion, and internal states (such as exploration, anxiety, etc.). The stimuli used in these experiments are not anticipated to be harmful or cause suffering (e.g. visual, acoustic, etc.). The expected impact of our manipulations will primarily be alterations in their social behaviour, hence we do not foresee any moderate or severe suffering for the animals.

In some experiments, fish will be exposed to subthreshold noxious stimuli (i.e. stimuli that do not cause any physical damage but activate nociceptors). For instance, an infrared laser will be used to heat a specific area of the fish's skin. The intensity of these stimuli will be tested on a small test cohort (< 5-10 fish) to ensure it remains subthreshold. Fish typically exhibit a stereotyped response, such as escape, to noxious stimuli before any physical damage occurs. The health of the fish will be continuously monitored.

To better understand the impact of specific genes on social behaviour alterations, we will also study changes in sleep behaviour and the effects of sleep deprivation. Although sleep deprivation can increase stress levels, recovery is usually complete following an initial period of deeper sleep.

In order to study how specific neurons process social information, we will image the neuronal activity of some fish with high-resolution microscopes. Zebrafish's transparency during early stages of development allows for non-invasive brain imaging. To achieve single-cell resolution, fish will be restrained in an agarose gel matrix. Literature suggests that this causes only mild stress, with fish displaying normal behaviours once freed.

In certain experiments, elements of the neuronal social circuit will be removed to verify the causal role of key neurons in processing visual, social, or somatosensory responses. These experiments are crucial to prove the direct involvement of a specific brain area in a social function or behavioural output and will only be performed after substantial evidence has been collected.

Larvae and juveniles will be exposed to small compounds that can either induce or rescue changes in sociality, sensory stimuli threshold, and sleep. In the few cases where we use drugs with a less clear pharmacological profile, we will first run pilot tests using a small number of animals to determine the lowest effective dose.

At the conclusion of these experiments, the larvae or juvenile zebrafish will be euthanized using a schedule 1 method, as approved by the Home Office.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Although the severity level for most of our procedures is classified as moderate, the majority of the fish used in our experiments will only experience mild severity (around 95%). In addition, larval and adult fish are continuously monitored by the researcher throughout the experiment, and additional health parameters such as blood flow, heart rate, and breathing are measured when image quality allows.

This constant monitoring allows any accidental harm or distress, such as slower or delayed behavioural responses, to be swiftly detected and the experiment terminated.

Based on our past experiments, we anticipate that fewer than 5% of the animals exposed to small molecules, imaging protocols, sensory stimulation, generation of transgenic or mutant lines, or cell ablations will exhibit moderate levels of severity, such as haemorrhages, seizures, or developmental disorders. In these cases, the experiment is immediately terminated, and the affected animal is humanely euthanized using approved Schedule 1 methods.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This research project aims to comprehensively understand the social brain network, which includes numerous structures scattered throughout the brain. To do this, we need to observe brain activity and behaviour in a live animal exposed to social or other sensory information. This is critical to understand how these brain areas are established and process social information throughout development.

Further, to comprehend how certain mutations or environmental factors influence the development of this network, we need to be able to track and image the entire circuit in a living animal.

Once we've identified specific neuronal populations and genes, we could begin exploring alternative non-animal model systems. Promising options include brain organoids, which are three-dimensional tissue cultures derived from stem cells, and computational models. Both methods could provide further insights into the functioning of the social brain network. However, until we've gathered sufficient foundational knowledge, in vivo research using animal models remains an essential part of our investigative approach.

Which non-animal alternatives did you consider for use in this project?

This research project investigates sociality, a characteristic inherent to whole organisms that cannot currently be replicated or studied adequately in non-animal models. As social brain areas are dispersed throughout many regions of the brain, our understanding of this intricate network requires a holistic approach that non-animal alternatives cannot yet offer.

Moreover, the use of non-vertebrate animals is unsuitable for our research. This is because non- vertebrates lack the evolutionary conservation and complexity of social behaviours we seek to understand, qualities essential to generating high-quality data that can be translated to humans. Zebrafish, a vertebrate model, are more similar to humans in

terms of brain structure and function, thus providing a more relevant and informative model for our investigations into the mechanisms underlying social behaviour.

Why were they not suitable?

This research is focused on examining complex social behaviours and their associated neural networks, features that are not adequately replicated in non-vertebrate models such as flies and worms. These organisms lack the evolutionary conservation and complexity necessary to accurately represent the social behaviours we aim to study.

Further, at this point in scientific advancement, there are no non-animal models available that can effectively mimic the entire gamut of social behaviours and their intricate underlying neural circuits. Therefore, our research requires the use of vertebrate models, specifically zebrafish, to garner a comprehensive understanding of the mechanisms that drive social 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?

This research proposal estimates the requirement of approximately 698,000 zebrafish over a span of 5 years. These zebrafish will be allocated for various tasks:

Maintenance. Maintenance of current genetically modified lines and creation of new ones: The team keeps larvae in shoals of 40-50 per tank for their studies on sociality. A total of 180,000 adult zebrafish are estimated for maintaining 6 generations of 80 distinct genetically modified lines. For the generation of around 35 new genetically modified lines, an additional 2,000 adult zebrafish will be needed. The team will also require 1,000 embryos for freezing sperm from important lines. This totals to 183,000 adult zebrafish over the next 5 years.

Behavioural experiments. The majority of zebrafish will be used at larval and juvenile stages. They will be imaged individually or in high-throughput tests (e.g., social and sleep tests), allowing up to 96 fish to be imaged simultaneously. This methodology will also be used to assess various compounds. Projecting from past experiments, the team anticipates tracking approximately 490,000 animals over the 5-year period.

Imaging experiments. Additional experiments will image brain activity during social behaviour, sleep, and following the removal of specific cells shown to be crucial for certain behaviours. About 3,000 animals will be needed each year for these experiments, leading to a total of 15,000 over the next 5 years.

In summary, the total number of zebrafish needed over the 5-year period will be 698,000, including 183,000 adult zebrafish for maintenance, and generation of genetically modified

lines, 1,000 for sperm freezing, 490,000 larval animals for behavioural tracking experiments, and 15,000 larvae for neural imaging studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

This text outlines the strategies that the we intend to use to reduce the number of zebrafish required for their experiments to the minimum possible:

Statistical Power Calculations: We will conduct statistical power calculations and utilize other statistical methods to determine the minimum number of fish required to yield significant results per experiment. This is always done prior to any experiment. Having conducted similar behavioural experiments over the years, they have a good understanding of the variability between fish which aids in these estimations.

Advanced Imaging Methods: We will employ advanced imaging techniques that can capture higher quality and larger amounts of data with fewer fish. This approach maximizes the value of each fish used in the study, reducing the overall number needed.

Pre-emptive Analysis: We will analyse the results of recorded experiments before conducting additional ones. This strategy enables them to re-evaluate and adjust the number of fish required to obtain statistically significant data for any particular experiment.

In summary, we are committed to reducing the number of animals used in their research by implementing robust statistical methods, employing advanced imaging techniques, and regularly reviewing experimental data to ensure they use only the minimum number of animals necessary.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

This text details the measures already in place at the fish facility to minimize the number of zebrafish generated and used:

Efficient Breeding Practices: The fish facility has instituted several procedures designed to ensure that the number of animals bred is the smallest possible to maintain stock lines and carry out experimental procedures.

Efficient Generation of Mutant and Transgenic Zebrafish: The facility has refined its processes for generating mutant and transgenic zebrafish to a point where they only produce the minimum number of fish necessary.

Early Identification of Mutants or Transgenics: Techniques such as fin clipping at early developmental stages are used to quickly identify mutants or transgenics, reducing the need for a large number of fish.

Maintaining Lines in Mutant Backgrounds: By maintaining lines in mutant backgrounds, the facility can reduce the total number of fish required.

Selective Raising of Founder Fish: To generate a large number of mutants efficiently, the facility utilizes deep sequencing techniques prior to five days post fertilization. This allows

them to selectively raise only those founder fish that harbour mutations of interest, thereby minimizing the number of animals used.

These measures exemplify a commitment to the principles of the 3Rs (Replacement, Reduction, Refinement) in animal research, ensuring that the number of zebrafish used is kept to a minimum, while maintaining high standards of animal welfare.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 text provides an explanation for the choice of zebrafish as the primary animal model for the research project due to their suitability and advantages for the specific experimental procedures planned:

Genetic Flexibility: Zebrafish are a simple vertebrate system that allows both forward and reverse genetic approaches to study behaviours such as social behaviour. This flexibility makes zebrafish a useful model for studying the genetics of complex behaviours.

Brain Similarity: Many zebrafish brain structures are similar to those of humans, which makes them a useful model for studying neurobiological processes that may be relevant to human health and disease.

Optical Transparency: Zebrafish are transparent, which allows for non-invasive imaging of brain activity throughout the entire brain with single-cell resolution.

External Fertilization and Development: Zebrafish eggs are fertilized externally and their development occurs outside the uterus, allowing researchers to follow the development of the entire brain from fertilization.

Genome Manipulation Efficiency: The genetics of zebrafish allow efficient manipulation of the genome, reducing the number of animals needed for certain experiments compared to other models.

Compound Administration: The ability of zebrafish to absorb compounds through the water makes them an ideal model for high-throughput experiments, including those involving environmental manipulations.

Minimal Animal Suffering: The research project mainly involves non-invasive tracking of zebrafish larvae and juveniles, which is not expected to cause any suffering to the animals.

For all these reasons, the zebrafish is a highly valuable model system for studying many aspects of biology, both developmental and behavioural, found in amniote vertebrates such as mammals.

Why can’t you use animals that are less sentient?

This text highlights the rationale for choosing zebrafish as the preferred model for the research project, focusing on their evolutionary simplicity as vertebrates and the presence of complex social behaviours. Here are the key points highlighted:

Suitable Complexity: Zebrafish are the least evolutionarily complex vertebrates that still exhibit the rich social behaviours the researchers want to study. This balance of complexity allows for more direct investigation into the underlying biological processes while maintaining relevance to higher organisms.

Established Protocols: The laboratory has already established methods for studying social preferences in juvenile zebrafish. Therefore, the majority of experiments are designed for these juvenile stages.

Limited Social Preferences in Larvae: Larval zebrafish do not exhibit strong social preferences, which makes them less suitable for social experiments. However, they are still useful for other types of experiments.
Use of Larvae for Other Experiments: Despite the limitations in studying social behaviour in larval zebrafish, these younger stages are still used in other aspects of the research, such as testing compound concentrations or identifying thresholds for somatosensory stimulations.

In conclusion, the researchers have chosen zebrafish because of their evolutionary simplicity, the ability to display complex social behaviours, and the adaptability of their different developmental stages to various experimental setups.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are committed to refining our procedures to minimise the welfare costs for the animals involved in our research. We have established a comprehensive monitoring system where the animals are continuously tracked using video cameras linked to computers. This enables us to remotely observe the animals for any adverse outcomes, ensuring quick response to any detected distress. Additionally, we are exploring automated software that can recognize unusual movements, indicative of distress, and alert the staff for immediate action.

For breeding genetically modified animals, we acknowledge the need to refine current procedures for DNA sample collection. Currently, we use a fin clip method, but we are actively investigating less invasive alternatives. There are promising techniques under development that involve skin swabs.

Should these methods prove to be reliable and efficient, we intend to adopt them, further reducing any potential stress to the fish.

In our imaging experiments, we maintain a stringent observation protocol. Any alterations in basal calcium imaging levels or atypical responses, such as higher than usual responses, are promptly monitored and addressed. This ensures we catch any signs of discomfort early and can adjust our procedures accordingly.

To ensure these refinements are effective, we will continuously evaluate and adjust our procedures based on feedback and findings from our continuous monitoring and from the wider scientific community. Our goal is to ensure that we maintain the highest standards of animal welfare while still achieving our scientific objectives.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We are committed to conducting experiments in the most refined way by adhering to well-established best practice guidance. To ensure the highest standards of animal welfare, we follow the principles of the 3Rs (Replacement, Reduction, and Refinement) as elaborated by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

In addition to the principles of the 3Rs, we rely on published best practice guidance from reputable sources. Our research adheres to the "Good research practice policies and guidance" provided by UK Research and Innovation (UKRI) and the Home Office Documents. These documents outline essential guidelines for ethical and refined animal research, covering various aspects such as experimental design, procedures, care, and the reduction of animal numbers.

We regularly review and update our procedures in light of advancements in best practice guidance to ensure that we are following the most up-to-date and refined methods. This includes keeping track of relevant scientific literature, attending conferences and workshops, and staying connected with the scientific community to stay informed about the latest refinements in conducting experiments.

By actively incorporating these best practice guidelines into our research, we aim to achieve the highest level of refinement and animal welfare while still achieving our scientific objectives.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have set up a system to ensure that we are always informed about the latest developments in the 3Rs. We hold regular meetings with an NC3Rs officer who keeps us updated on new advances, tools, and techniques in the field. Furthermore, our facility employs dedicated staff who are responsible for developing online and in-person training refreshers. All our team members are required to attend these sessions at least once a year, ensuring continuous learning and application of the 3Rs principles in our work.

In addition, to implement these advances effectively during the project, we will incorporate new techniques or methods as soon as they are validated and deemed appropriate for our study. For example, if a new method reduces the number of animals required for certain experiments or if a new non-invasive imaging technique becomes available, we would adopt these new practices immediately. We also plan to allocate time during our team

meetings to discuss and brainstorm how we can effectively apply these new advances in our project.

Furthermore, we have a system in place for regularly reviewing and updating our experimental procedures and protocols to ensure that they reflect the most current 3Rs practices. This includes regular audits of our practices and seeking external advice or review when necessary.

76. Tissue control of T-cell immunity

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, Immunology, Lymph node, T cell, Dendritic cell

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 determine what signals in tissues control and direct immune responses and to understand how this goes wrong in 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?

Immunotherapy, where the body's own immune defences are used to combat cancer, can lead to dramatic results and even functional cures for many patients with advanced cancer, but currently only a minority of patients receive any benefit at all.

Most of these therapies work by activating specialised immune cells called T cells which kill infected or cancerous cells. During cancer or infection, T cells are first activated in lymph nodes before moving into tissues to kill target cells. In cancer, T cells lose the ability to kill these cells and this is what immunotherapy seeks to restore.

The type of immune cells within the tumour and the consequences of this for the lymph node where T cell responses are initiated are key factors in determining whether a patient is likely to respond to this type of therapy. It is therefore important to understand how tissues normally communicate with lymph nodes and how this goes wrong in cancer to understand why some patients don't respond and to potentially develop new approaches to increase the applicability of these therapies.

As T cells behave differently in different tissues it is also important that this work is carried out in a range of organs including lung, gut and skin.

What outputs do you think you will see at the end of this project?

This project is likely to lead to a greater understanding of how the immune landscapes of tumours are determined and should allow us to test candidate approaches to alter this leading to improved responses to immunotherapy. We should also better understand how infection alters the lungs and how this alters future tumour development which has important implications for public health. Finally we also should better understand how tissues communicate with the lymph node to ensure appropriate immune responses are initiated. This final point will also give us a better understanding of what goes wrong during cancer leading to future work where we can try and fix these problems to improve anti-tumour immunity.

It is expected that these individual areas of study will lead to several publications sharing knowledge with the broader scientific community.

Who or what will benefit from these outputs, and how?

In the short term this work will benefit the research group by furthering our understanding of the fundamental processes ongoing within the tumour microenvironment and understanding how this leads to lymph node dysfunction. This will also be of use to our scientific colleagues as many groups are utilising immunotherapy to treat cancer and this additional knowledge will inform potential rational combination therapeutic approaches. This work also has implications for our understanding of autoimmune diseases and immune tolerance and so will provide insight to researchers in those fields.

Longer term, by informing these areas this work may lead to palpable patient benefits by informing the creation of rational therapeutic approaches seeking to influence the immune composition of the tumour or to improve lymph node function in the context of cancer.

How will you look to maximise the outputs of this work?

Our work will be published with open access agreements to ensure broad dissemination of findings. We will present at national and international conferences where possible. We will share experiences of unsuccessful approaches with colleagues, collaborate with other relevant research groups and make any large datasets publicly available.

Species and numbers of animals expected to be used

- Mice: 30,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using adult mice for our studies because we are studying how immune responses are generated in complex organs like the lung or the gut. As such we cannot use purely cell line based systems as they don't replicate the complexity found in an organ and we cannot use non-protected animals as we need a vertebrate model (and really a mammal) so that they have an immune system similar to the human one. Mice also have been used extensively in immunological and cancer research meaning that there are types of mice which can be used to unpick complex questions carefully which are not available in other species.

Typically, what will be done to an animal used in your project?

A typical mouse on this license will have an ear notch made for identification, it will then be given a respiratory infection, by putting a drop of liquid on its nose which it subsequently inhales, or a chemical or cell injection will be given to initiate development of a tumour, either within the lung, the gut or on the side under the skin. Most infections or tumours will occur over the course of 1-2 weeks, although some longer tumour models may be slower to develop taking up to 3 months (where the majority of the time mice will show no signs of tumour development). During this time the mice may be administered drugs to modify the immune response to the infection or tumour and this can occur multiple times, although not more than once a day and not more than 16 times total. At the end of an experiment a mouse may be anaesthetised to allow imaging of the tumour or infected site, but it will not be allowed to recover from this procedure being killed by a schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

During infections mice are expected to lose weight and show some clinical signs of illness including some degree of staring coat, hunching and reduced mobility. This is expected to last for about 3 days and then they are expected to recover.

For lung tumours, mice may show some difficulty breathing at the very end of the experiment and may lose some weight. This would occur in the last day of the experiment and would constitute an end point.

For bowel tumours mice may lose weight and show reduced mobility, but this would be at the end of tumour development and would constitute an endpoint, so it would last up to a day.

For subcutaneous or mammary tumours there should be no adverse effects behaviourally or clinically, however, the presence of a tumour will constitute a moderate severity procedure due to its size.

These adverse effects will be monitored throughout experiments to ensure suffering is minimised in achieving the aims.

Expected severity categories and the 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 on this license will be mild or subthreshold when in breeding/maintenance or in infection protocols although a proportion may lose enough weight to constitute moderate severity (<25%). For subcutaneous tumours the majority of mice (>80%) will experience moderate severity due to the expected size of the tumour, however, this should cause no other clinical signs. For lung and colon tumours most mice will reach endpoint (>80%) and so would also experience moderate severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are studying how immune responses are generated in complex organs. As such we cannot use cell line systems as they don’t replicate the complexity found in an organ and we cannot use other animals as we need a vertebrate model (and really a mammal) so that they have an immune system similar to the human one.

Which non-animal alternatives did you consider for use in this project?

We have considered, and will use to a limited extent, cell line based assays measuring signals which can direct cell movement. We have also looked at using cell lines which can be used rather than using cells derived from mice.

Why were they not suitable?

The issue with these systems is that they do not provide real insight into what happens in tissues and cannot be used to explore how changes in tissue which occur with infection or tumour development affect these responses. Cell lines also don't behave like normal cells derived from mice and so, again, can only be used for a small number of experiments where this can be controlled for.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are based on previous experience of use from our previous project licence. These numbers provide enough opportunity to properly interrogate the processes involved in our scientific questions in a way that provides robust data ensuring we are not underpowering studies and thus wasting mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will minimise numbers in a few key ways, informed by use of the NC3R's Experimental Design Assistant:

Ensuring we use the fewest number of animals to show a significant response

Getting as much information from every experiment by taking multiple tissues

Making sure we don’t breed more mice than we need

Developing ways of answering questions with cell lines where possible

Where possible utilising less complicated genetic systems which require fewer mice to be bred

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 set up breedings with the optimal genetic backgrounds to get the highest proportion of usable mice wherever possible.

We will share tissues with collaborators and within the lab to get as much use as possible from every mouse.

When embarking on a new approach we will use small groups initially to estimate the effect size and to allow appropriate power calculations to be performed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The laboratory mouse is the species of choice for studying immunology for several reasons:

Despite the significant differences that exist between mice and humans, decades of basic science using laboratory mice strains have shown that findings in mice are often relevant to humans and that they also help to reveal general principles that regulate biological systems. There are similarities at all levels from genes through to organs between the human and mouse immune systems strengthening the validity of using mouse models.

Furthermore, there is a wide range of genetically modified mice allowing specific questions to be asked which would not possible with other vertebrates. Importantly for us, there exist mice engineered to only produce specific types of T cells allowing us to accurately study immune response. Other examples of mouse strains we will use mark specific immune cell types so we can study their localisation or mice lacking specific genes which allow us to understand the roles of particular cells and proteins. Finally, we will use mouse strains that are predisposed to develop specific cancers for studying the contribution of the microenvironment on immune priming during cancer development.

Tumour models

We will inject tumour cell lines into mice either under the skin, into the blood stream or into the gut wall where they can develop into tumours in mice with intact immune systems. The fact that these tumours can grow alongside the immune system make them the best models to study suboptimal T cell responses and mimic immune responses to cancer in human. Tumours are monitored in site specific ways with gentle palpation of skin tumours, tracking breathing in mice with lung tumours and endoscopy for mice with tumours of the bowel.

To minimise suffering, when possible, organs will be harvested early and before tumours spread extensively and before any adverse clinical signs are apparent. Unfortunately, the time course of tumour development and its subsequent spread is not entirely predictable and experimental manipulations also alter timing in ways we cannot always anticipate.

We are also using well-established systems to induce tumour formation using viral, chemical or genetic manipulations that are standard in the field and which we have extensive experience of monitoring.

All models are the most refined for different purposes. Transplant models allow for assessment of specific T cell responses while the other systems of tumour induction are slower and so allow us to study how tumours interfere with the immune system over time in a way more representative of human tumours.

Infectious models

We have carefully chosen 2 respitatory viruses as infectious models. The immune response to both is well characterized and relies on T cells. There are also mutants available that carry specific proteins that are recognized by specific T cells, or that allow us to identify infected cells. Furthermore, it is known that viral infection provides a model of optimal T cell priming as a contrast to that seen during tumour development and this makes these ideal models for investigating the deficiencies in the cancer environment.

Furthermore we have shown that lung infection leads to long term changes in the lung relevant to tumour development and so we will use these models to understand how these changes may lead to impacts in tumour development.

Why can’t you use animals that are less sentient?

The immune system of the mouse has many similarities to that of a human being a mammal. Other less sentient, non-mammalian potential models have very different immune systems as compared to humans making their use inappropriate.

Adult mice are required because the immune system does not develop fully until adulthood and as such earlier developmental stages are not appropriate for these studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will refine the procedures we carry out in multiple ways and will continue to further refine our procedures by engaging with colleagues to discuss best practices and through reading the relevant scientific literature. Currently we refine, and plan to continue refining, our protocols in a range of ways listed below.

We will pay careful attention to animal husbandry and provide environmental enrichment and co- housing to avoid social isolation. The animal facility is well equipped and the staff are experienced and well trained. These factors will ensure appropriate conditions for the mice are maintained at all times.

When possible, we will use mouse models which can be manipulated to only affect specific cells or to only occur for limited time periods to minimize any adverse effects.

We will make every effort to minimize the number of procedures per animal. For example, given our experience with vaccination, we will choose precise time points to sample blood, reducing the amount of pain, stress and suffering that the animal is likely to experience.

When administering drugs, we will follow guidelines in the field to ensure only appropriate amounts are administered (these guidelines written by Workman can be found in 'Guidelines for the welfare and use of animals in cancer research'). Drugs that have not previously been used in mice will only be used when there is good reason to believe that they will be well tolerated and the minimum amount will be used. If necessary, we will first perform a pilot test using a small number of animals to ensure no adverse effects develop.

For immunization experiments, we will use adjuvants that are effective but cause minimal adverse effects.

The infections listed under this protocol have been used extensively in experimental research and the effects of the doses we will use are well studied. We will use doses which induce only temporary and moderate suffering in the mice, similar in severity to a flu infection in humans. We will increase the dose for any subsequent infections, but mice will have developed immune memory, similar to that against a vaccine, and will eradicate the pathogen very efficiently. We will also use the least aggressive versions of any infections which can be used to generate the data required.

Appropriate techniques will be used during surgery to minimise adverse effects. Animals will be monitored before surgery to ensure that they are fit for the procedure, during the surgery to confirm that they are deeply anesthetised (sufficiently to feel no pain) and post surgery to ensure they recover well. Animals will be kept warm and hydrated throughout the procedure and appropriate analgesia (pain relief) will be given throughout the entire procedure and following surgery as required.

For mice irradiation, we will split of the doses of radiation in 2 separate doses separated by at least 3 hours to minimize side effects. We have also replaced most irradiation with busulfan pre-treatment which is associated with fewer adverse effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the Workman "Guidelines for the welfare and use of animals in cancer research" to ensure we use best practice with all our tumour models.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will engage with the AWERB culture of care and local animal users' group meetings to learn about advances.

We will also continue to engage with the literature as we have previously which led to the adoption of busulfan as opposed to irradiation and the use of the urethane model of lung cancer to replace some of the genetically engineered models previously used.

77. Genetic and developmental origins of congenital malformations

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

DNA mutation, congenital disorders, craniosynostosis, male germline, pathogenic mechanism

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project will investigate DNA mutations causing human congenital disorders and study disease mechanisms, with a focus on developmental abnormalities of the craniofacial region (skull and face).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The proposed work will form a major part of our team's research programme to identify and characterise new candidate genetic causes of human congenital disease, and assess

the origins, mechanisms and consequences of mutations for development. Our main disease focus is craniofacial malformations, particularly craniosynostosis, the premature fusion of one or more of the cranial sutures of the skull. This is a serious disorder, affecting ~1 in 2,000 children that is associated with multiple complications affecting hearing, vision, breathing, dentition, feeding, cognition and neuropsychological development. Most problematically, it can be associated with restriction of brain growth and raised intracranial pressure which can be life-threatening. The mutations that cause these disorders most often occur to unaffected parents (de novo mutations) via spontaneous mutational events originating in the male germline. In the course of our human genetics studies we often identify candidate DNA mutations that are hard to prove as the cause of the disorder. It is therefore important to undertake this work for three reasons: 1) it will provide evidence to support or disprove the role of specific DNA mutations in congenital malformations, 2) it will increase understanding of the origins of the causative mutations that fuel the ongoing occurrence of genetic disease in the population, with a particular focus on mutations arising in the testes, and 3) it will illuminate the pathophysiological mechanisms by which such mutations cause craniofacial malformations. This information is extremely valuable to families as there may be specific complications to screen for, as well as management and treatment implications. In addition, it may end a diagnostic odyssey and open doors for extra support. Importantly, a genetic diagnosis means the family can receive accurate counselling on recurrence risks and genetic testing. From the research perspective the work will generate new and detailed information about mutation origin in the testes, and about normal and abnormal skull development which is essential for future prevention and treatment strategies.

What outputs do you think you will see at the end of this project?

New information

The establishment of solid evidence for the causal role of candidate mutations in craniofacial malformations.

Increased understanding of the pathogenic mechanisms that lead to abnormal craniofacial development.

The placement of a newly validated disease gene into a pathophysiological framework is essential for a complete description of the processes that cause craniosynostosis.

Identification of new mutations in their tissue of origin, i.e. the male germline (the testes), allowing us to understand why and how often they occur.

The generation of robust models for further mechanistic investigation, for example of non-coding variation in human disease.

Publication and dissemination

We intend to publish key findings from these analyses in journals specialising in genetics or developmental biology and have a track record of doing so.
We will make mutant strains of interest to the wider research community.

Who or what will benefit from these outputs, and how?

Based on this work we hope to obtain evidence supporting or refuting the involvement of a specific genetic mutation as a cause of craniosynostosis or other craniofacial malformation in patients. The new knowledge will benefit patients and their families in the short to medium term. This will be through our collaborations with clinical geneticists and genetical counsellors who will disseminate this improved information so that patients with genetic mutations receive accurate advice and can take informed decisions during reproduction.

More broadly our work will provide information on the how the skull and face develops which will benefit other scientists and clinicians investigating craniofacial malformations in the medium to long term. In addition, our studies will be of general interest to the scientific community as they will add to the understanding of how mutations arise, which is important to predict how our genomes evolved and why genetic diseases occur.

How will you look to maximise the outputs of this work?

We are in collaboration with a large consortium of researchers that bring clinical genetics and mouse genetics together to better understand and treat human diseases. This enables us to maximise our outputs by the sharing and dissemination of animal models and tissues. In addition to the consortium, we disseminate our findings through presentations at National and International conferences and through publications in relevant open access journals.

For patients and their families, we are in collaboration with a wide network of clinical geneticists and genetic counsellors with whom we share new knowledge. This information is also shared with patient groups and charities associated with craniofacial disease.

Species and numbers of animals expected to be used

- Mice: 6800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Arguably the best model organism for studies of the skull is the mouse because the cranial suture pattern is very similar to humans, it is small in size and has a short reproductive cycle, the genome/transcriptome is well characterised, and there are highly developed technologies for genome manipulation. Also the embryological origin and identity of the calvaria and cranial sutures is clearly understood in the mouse, and more similar to human than non-mammalian species. Primarily our work will model human DNA variants that cause craniosynostosis (the premature fusion of one or more sutures of the skull) in the mouse. As craniosynostosis most often occurs before birth much of our analysis will be performed at embryonic stages. Some mice will be analysed when the skull has stopped growing (3-8 wks).

Typically, what will be done to an animal used in your project?

Genetically altered mice will be bred by natural methods. The mutations that occur in our strains are not expected to cause any pain that is more than mild and transient.
Some mice will undergo a tissue biopsy of the ear for identification, with the surplus tissue being processed to allow genetic identification of the mouse.
Rarely mice will develop overgrown incisors due to craniofacial malformation. Scanning shows that overgrowth is limited to the incisors because of facial suture fusion and that premolars and molars are unaffected. These mice will undergo teeth trimming on up to 10 occasions under light anaesthesia.

Some mice will experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal).

What are the expected impacts and/or adverse effects for the animals during your project?

Rarely craniofacial lines may have dental malocclusion and incisor overgrowth. In such lines, affected mutant animals may need teeth trimming under light anaesthesia to maintain nutrition. They are expected to recover quickly and will be given painkillers just like people recovering in hospital.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severity	Mice
Severe	0%
Moderate	5%
Mild	5%
Sub threshold	90%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To establish that a DNA mutation causes craniosynostosis or to delineate how its leads to an abnormal skull, direct study of the effects of the mutation on cellular behaviours in intact cranial sutures is necessary. Cranial sutures are complex 3-dimensional structures containing multiple cell types interacting and communicating with each other. Sutures need to remain patent and enable growth of bone over several weeks, during which their size increases by several orders of magnitude. At present there is no artificial system able to

recapitulate these complex developmental processes. Methods have been developed to isolate cranial sutures from embryos and maintain them in culture for 1-2 weeks, however this protocol removes both the normal biomechanical forces on the suture and the normal supply of nutrients and oxygenation so does not replace in vivo studies.

Which non-animal alternatives did you consider for use in this project?

As an alternative we have considered the use of organoids. Currently, about two-thirds of our programme of work either utilises human DNA samples, which are employed for all the genetic screening, or cell lines and in vitro models, which are used to establish the molecular details of pathogenesis.

Why were they not suitable?

To investigate what leads to abnormal craniofacial growth it is necessary to have all the components of this complex system together, including underlying brain growth and expansion which drives growth of the skull via multiple different cell types and tissues. Currently an organoid system which could replicate this dynamic system does not exist. However, we continue to combine in vivo and in vitro approaches, and are currently analysing patient derived cell lines which have been induced to a relevant cell type to see if they provide an alternative to obtain functional evidence for pathogenicity.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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've used our annual return of procedures data to estimate the number of animals that we will need to use for breeding. For new strains, the estimated numbers of mice required are based on our experience over the last 6 years in generating eight new lines. For this Project we have allowed for the generation of 10 different new strains, and included provision for crossing onto a different background and production of homozygous genotypes. Calculations typically show that we need group sizes of 10 to achieve the quality of results we need.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When analysing new mutant strains, if available we will make use of animals available from storage banks. In cases where genetically modified strains of particular interest for scientific study are not already available, we will collaborate with expert teams licensed to generate appropriate genetically altered lines, which could be transferred to this project. For most purposes we would be seeking qualitative differences between mutant and wild-type littermates - for example do they have craniosynostosis? For this we calculated the

minimum number that would give an effect that is statistically significant, where difference in effect of the treatment between the two groups is relatively large.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Best practice guidelines will be used to manage breeding colonies, with particular attention paid to good breeding performance. The in-house database will be used to make decisions on which animals to use for breeding, and to control colony size so that only those animals needed for experiments are produced. Where possible we will maintain strains in the homozygous state so that fewer animals are required. Useful strains will be preserved so that they do not need to be made independently by other researchers in the future. Tissue will be shared with collaborators throughout research network, and on request.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The models chosen are those that best recapitulate the phenotype of the equivalent human disorder (for example, craniosynostosis) most reproducibly. Most of the craniofacial strains of mice that we have generated have rarely posed any specific husbandry problems, however an issue that rarely occurs is incisor overgrowth. In these cases, only the animals of particular scientific value are kept, for the shortest possible amount of time, with teeth trimmed using the most refined method, and mice fed wet mash. In our breeding strategy we will use less affected animals to produce mice that need less interventions. Animals are maintained in the best physiological state through housing in individually ventilated cages to maintain clean conditions and reduce experimental variability. Our experimental methods are primarily the breeding of craniofacial strains of mice, with a small proportion being administered substances that allow us to follow individual cells in the offspring, or to assess the development of new mutations. These methods chosen are widely used and the most refined, yielding the most precise data at the earliest endpoint, to avoid or cause the least pain, suffering, distress, or lasting harm.

Why can’t you use animals that are less sentient?

Amongst model organisms (including chicks, amphibians, and fish), only mammals have a pattern of cranial sutures similar to these structures in humans. Amongst mammals, the mouse is the most suitable for intensive study because of its small size, short reproductive cycle, and the availability of well-developed genetic technologies to manipulate the mouse genome, and associated resources of genetic strains. Most of the work uses embryo stages, as the craniofacial malformations we work on develop during gestation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be regularly monitored as a new genetic line is established. The health of mice will be assessed regularly (at least 2-3 times/week) on the basis of their eating, drinking, movement, growth and general appearance and behaviour. Advice will be sought from the named veterinarian and the animal welfare officer about special healthcare and husbandry requirements (e.g., regular bodyweight/health checks, provision of suitable diet). We will employ a simple scoring system based on the observation of signs that would either trigger additional husbandry requirements or humane killing in animals that develop severe effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow best practise in conducting experiments, informed by our Named Information Officer or by reference to the following resources: www.nc3rs.org.uk; https://norecopa.no; https://www.lasa.co.uk.In addition, our close working relationship with experts in mouse genetics ensures we are exposed to current best practises in mouse research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To stay informed, we use the NC3R's website and review the newsletters and bulletins that are internally distributed by the internal 3Rs Sub-committee. In addition we attend internal 3R's meetings. Suggestions or advances that come out of these sources are discussed and implemented immediately if appropriate.

78. Preclinical assessment of prophylactic and therapeutic immune products 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

Immunotherapy, Viral vector, Nanoparticle, Cancer, Immunology

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to demonstrate functionality and efficacy of novel immunotherapy products (e.g., viral vectors, synthetic peptide nanoparticles), designed for the treatment and prevention of infectious disease, autoimmunity or 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 core mission of the establishment is to discover and develop novel immunotherapeutic products for the treatment and prevention of infectious diseases, autoimmunity and cancer. Conditions will be targeted where there is currently unmet patient need; meaning that therapies are either not available or they are not sufficiently effective. The project will

generate data to support "Go / No-Go" decisions for these product types as they are in the preclinical development stage.

Chronic infection with hepatitis B virus (HBV) is an example of a condition that has been targeted by our establishment. Chronic HBV infection is a global public health challenge on the same scale as tuberculosis, human immunodeficiency virus and malaria. The current prophylactic vaccine has no effect on established chronic infection. Available treatments suppress viral replication, but they are not curative, due to the persistence viral components in the liver and the failure of chronically infected patients to mount an immune response that is sufficient to clear the infection. Thus, in most cases, treatment must continue for life. Furthermore, even successfully virally suppressed patients may still develop liver cancer. Our treatment is designed to generate high magnitude T-cell responses and also generate lasting cell mediated immunity with the aim of complete clearance of HBV from patients.

What outputs do you think you will see at the end of this project?

This project will comprise a series of research work packages aimed to design, develop and optimise immunotherapeutic products (e.g., viral vector-, peptide nanoparticle-based) targeting infectious disease, autoimmunity and cancer where there is currently unmet patient need. The expected outputs are the generation of critical preclinical data to identify candidate products that are most likely to have clinical potential and support clinical trial applications. As such, this project will support the progression of the best candidate products through the establishment's product development pathway with an ultimate goal of patient benefit.

Who or what will benefit from these outputs, and how?

Knowledge generated from this project will provide valuable information that will enable scientists involved in the establishment's internal product development activities to design effective immunotherapeutic products. Combined data from the work packages completed under the project will help to build overall knowledge and experience, enabling increased efficiency/probability of success for future products in early development. The goal is to progress immunotherapeutic products through clinical development to market resulting in significant patient benefit.

How will you look to maximise the outputs of this work?

Both negative and positive data will be disseminated internally to inform future design and optimisation of immunotherapeutic products. Where appropriate (from a commercial/Intellectual Property perspective), data will be published in scientific journals and/or presented at scientific meetings to disseminate knowledge to the wider scientific community.

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.

Young adult mice (i.e., 2 to 4 months old) will be used for the study. We have used mice in our immunogenicity studies previously as they have an intact immune system and have been demonstrated to respond to our immunotherapeutic products. The mouse immune system is considered mature at 8 weeks of age, so we believe an age of 8 to 10 weeks to be appropriate for our studies. Using mice of this age also means that both male and female mice may be utilised in the studies, as opposed to excluding male mice due to excessive fighting in later stages of development. This uses purpose-bred mice more efficiently than single sex studies and generates more balanced data sets.

Typically, what will be done to an animal used in your project?

Animals will be housed in colonies. When the study commences, mice will receive a dose of an immunotherapeutic product by injection through intramuscular, intravenous, intraperitoneal or subcutaneous route. Mice in some experimental groups will receive a second and/or third injected doses of a product intended to boost the immune response to the antigens delivered by the immunotherapeutic.

In some experiments, non-invasive *in vivo* imaging will be conducted following administration of the immunotherapeutic. In this procedure, mice will be anaesthetised with inhalation anaesthetic and placed inside the imaging equipment for a maximum of 30 minutes. Mice will be subjected to a maximum of two *in vivo* imaging procedures per week, with at least 48 hours between instances. Any individual mouse will receive a maximum of six instances of general anaesthetic in total.

In some experiments, small volume blood samples may be taken from conscious animals via superficial blood vessels. Any individual mouse will be subjected to a maximum of one blood sampling per week, and five instances of blood sampling.

Where products designed to target specific cancers have been shown to be suitably immunogenic, mice may be inoculated with tumour cells that will lead to the development of tumour tissue in the mice. Immunotherapeutic product will either be given pre-inoculation (to test for prophylactic efficacy) or post-inoculation (to test therapeutic efficacy) with tumour cells. In these experiments, live animal imaging (as described above) may be used to visualise the location and level of tumour cells in the mice. Subcutaneous tumour volumes may be measured using callipers up to twice a week.

At the experimental endpoint (usually Day 28 for immunogenicity studies, but this may be longer for studies where tumour cells are given), mice will be humanely killed, and blood/relevant tissue (usually spleen) will be removed to conduct experimental procedures in the laboratory.

What are the expected impacts and/or adverse effects for the animals during your project?

Injection of the immunotherapeutic product will cause mild, transient pain in the mice, but no lasting harm. Intramuscular delivery can be difficult in mice due to the small muscle

mass available for injection and so mice will be briefly anaesthetised using inhalation anaesthesia prior to administering the injection. This will prevent the animal from moving during the procedure to enable the animal technician to perform the delivery is as accurately as possible, thus minimising the risk that suffering will be greater than transient. Mice will be monitored to check for symptoms of pain at the injection site, such as abnormal gait or unprovoked vocalisation. The immunotherapeutic product is intended to generate (or moderate with respect to treating autoimmune conditions) an immune response. The dose will be set according to prior experience and so no adverse events are expected resulting from the activity of the immunotherapeutic product.

Where *in vivo* imaging procedures are conducted, adverse events are not expected since the occurrence of events due to anaesthetic use is very low in mice (<1%) where these are performed by suitably trained/experience personnel.
Blood sampling via venipuncture will cause transient minor discomfort. Adverse events from this procedure are rare.

In studies where tumour cells are given, mice may experience symptoms due to the growth of tumour tissues. This can include changes to physical condition and behaviour, problems with mobility, and weight loss. In some instances this may lead to ulceration of the tumour.

Expected severity categories and the 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 will receive up to three injections of a product intended to generate (or moderate with respect to treating autoimmune conditions) an immune response. Mice are expected to recover quickly from this procedure. In the majority of experiments, the delivery route will be intramuscular. Due to the small size of the target muscle in mice, there is a risk of Moderate severity in a small number of animals. It is estimated that no more than 10% of mice will experience Moderate severity.

Where *in vivo* imaging procedures are conducted, mice are expected to recover quickly from anaesthetic. No harm is expected as a result of this procedure. The expected severity is Mild.

Where blood sampling procedures are conducted, mice are expected to recover quickly from the procedure. All animals where blood sampling is performed will experience Mild severity.

Where tumour cells are given to mice, the severity is expected to be Moderate in around 25% of animals (i.e., mice from untreated control groups). This is because test animals will be treated with immunotherapeutic products where immunogenicity has been demonstrated and, therefore, control of tumour growth is likely.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animal models to understand whether immune responses are generated by the immunotherapeutic products being tested. Where immune responses are generated, the magnitude and quality of the response is important in assessing the suitability of drug candidates for progression through our product development pathway.

The generation of an immune response results from very complex biological systems in which many different cell types interact and communicate with each other. In addition, immune cells are in constant movement that allows them to interact with different cells and tissues at different stages of their development. These processes are so complex that currently no *in vitro* system can replicate this.

In addition, testing efficacy of immunotherapeutic products through use of tumour models can be best achieved in an *in vivo* setting.

Which non-animal alternatives did you consider for use in this project?

Laboratory-based cell culture systems were considered as an alternative. For example, for immunotherapeutics targeting cancer, there are increasingly sophisticated *in vitro* 3D models available (reviewed in Tosca, E.M. *et al.* Replacement, Reduction, and Refinement of Animal Experiments in Anticancer Drug Development: The Contribution of 3D *In Vitro* Cancer Models in the Drug Efficacy Assessment. Biomedicines 2023, 11,1058). These tumour models are relevant in that they comprise genuine human cancer cells. In instances where patient-derived material is available, relevance is further increased.

Why were they not suitable?

Wherever possible, *in vitro* techniques will be used to test immunotherapeutics as they go through preclinical development.

However, i*n vitro* methods are unsuitable to entirely replace the need to utilise animals in this project. Although there has been much improvement in recent years, cell culture systems are still not able to replicate the complex nature of immune and non-immune cells involved in the process of responding to how immunity is developed through the use of adenovirus and/or peptide nanoparticles.

An example of how animal use could be reduced (but not replaced) is the potential to generate immunogenic T cells in mice but test the activity of the cells *ex vivo* against relevant tumour models in cell cultures.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals that will be used for this five-year project have been estimated based upon the number of mice used in preclinical development over the last year. It is estimated that approximately 2,500 mice will be used over a five-year period for the assessment of immunogenicity of test products. A further 500 mice have been estimated for studies in which efficacy of immunotherapeutic product against tumour development will be tested.

e.g., An example study testing an immunotherapeutic approach where immune response is primed with one product and boosted 14 days later would require 30 mice.

Group 1 = PBS only (negative Control) n=10 mice Group 2= Prime only n=10 mice
Group 3 = Prime + Boost n=10 mice

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Use of inbred or outbred F1 strains of mice yields more reproducible results, which allows for a smaller number of animals to be used to gain statistically significant data.

Both male and female mice will be used in the studies, thus increasing efficiency in the breeding of mice relative to female-only studies performed previously.
All intended experiments will be planned using the Experimental Design Assistant to ensure that the experimental design yields meaningful data, and to reduce the numbers of mice used 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 appropriate, additional tissue (e.g., lymph nodes) will be harvested from mice and retained for use in additional analyses as might be required in the future.
Both male and female mice will be used in the studies, thus increasing efficiency in the breeding of mice relative to female-only studies performed previously.
Where relevant, pilot studies will be performed where immunotherapeutic products are significantly different from those already tested to ensure that design of experiments utilises the correct parameters (e.g., animal numbers, product dose).

The use of established Standard Operating Procedures to perform routine techniques will ensure reproducibility and reduce the possibility of unnecessary animal use due to poor procedure.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this project, laboratory mice will receive up to three intramuscular injections of an immunotherapy product typically at 2 – 4 week intervals. Intramuscular injections are used since this is the route that will be used in humans. The injections will cause mild, transient pain in the mice, but no lasting harm. The product is intended to generate an immune response. No adverse events are expected resulting from the treatment.

In some studies, mice will be subjected to *in vivo* imaging procedures to visualise distribution of the immunotherapy product in the mouse, and how long the product is present. Where *in vivo* imaging procedures are conducted, adverse events are not expected since occurrence of events due to anaesthetic use in mice extremely low. Indeed, the use of *in vivo* imaging procedures are designed to cause less suffering since live monitoring means that less mice can be used in a biodistribution study.

In the minority of experiments, illness (e.g., cancer) might be induced in mice to test the ability of the immunotherapy product to prevent or limit the development of disease. Performing these experiments will be strictly limited to instances where additional efficacy data is required to justify further development of the drug product. In these instances, mice will be closely monitored to ensure that the endpoint is as humane as possible.

Why can’t you use animals that are less sentient?

Mouse models are the least sentient and best characterised experimental models for use in the testing of products where an immune response is required. Adult mice (> 8 weeks old) have a fully developed immune system that is a good representation of human immunity. Work cannot be conducted in lower vertebrates, invertebrates, or cell lines due to the poor resemblance of these options to the clinical setting.

Considering less sentient model species, it is accepted that zebrafish (for example) can be useful models for human disease in some instances. For example, a recent review described zebrafish as a useful model for study oncongenesis and the development of cancer (Kwiatkowska I, et al. Zebrafish- An Optimal Model in Experimental Oncology. Molecules. 2022 Jun 30;27(13):4223). However, we concluded that zebrafish would not be suitable for the purpose of investigating immunogenicity or efficacy of our products. Delivery of our products into zebrafish and obtaining meaningful samples for testing would be extremely difficult to achieve due to the size of the organism. In addition, dosage cannot be easily translated directly from zebrafish into human doses due to differences in the physiology between the species.
Terminally anaesthetised animals cannot be used since the required immune responses takes several days to fully develop.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Prior to experiments starting and while experiments are in progress, I will be in regular communication with the Named Animal Care and Welfare Officer at the animal facility to ensure that the studies being conducted are as humane as possible. Discussion will take place after studies have been completed to discuss possible ways in which subsequent studies can be improved.

One method of improvement that will be introduced is the administration of anaesthesia prior to performing intramuscular injections. The purpose of this is that the animal will be relaxed meaning that there is less likely to be harm caused by injection into a relatively small muscle.

To reduce harm, it is standard practice to use a new needle for every injection performed on the experimental mice.

Where mice are inoculated with cancer cells, the use of bioluminescent imaging will allow non-invasive monitoring of tumour growth. By using bioluminescent imaging, the growth and regression of the tumour can be monitored to avoid unnecessary prolonged harm to the mice from the tumours becoming too big or too invasive.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

As Project Licence holder, I will consult PREPARE guidelines and utilise the NC3Rs Experimental Design Assistant as appropriate.

The animal facility is invested in the welfare of the mice housed there. The facility is both AAALAC and ISO9001-2015 accredited. In addition, LASA guidelines are routinely followed for in-life sampling and dosing.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As Project Licence holder, part of my role is to remain aware of advances in 3Rs and to ensure that experiments performed under the Project Licence follow current best practices. To remain appraised of advances in the world of laboratory animal science, I am a member of LASA and have a subscription to *Laboratory Animals* journal.

Our establishment is located on the same campus as the animal facility. This will facilitate an open route of communication with the Named Animal Care and Welfare Officer and will provide opportunity to discuss potential improvements during the project and implement the improvements, where this is appropriate.

79. Understanding the neurohormone regulation of mammalian reproductive physiology and disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Reproduction, Hypothalamus, Hormones, Infertility

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 first overarching aim of this work is to reveal how brain hormones regulate ‘healthy’ reproductive physiology and associated behaviours (e.g. sexual and social behaviours). A second more translatable aim of this research project is to identify novel targets and develop new treatments for infertility (e.g. ovulation disorders, hypothalamic amenorrhea, etc.) and related behavioural disorders (e.g., decreased sexual behaviour, decreased social interactions, etc.).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In developed countries, reproductive disorders are prevalent, affecting 15-20% of couples at some stage of their lives. In about a third of the cases investigated for subfertility, no specific cause can be identified, which limits treatment options. The underlying pathophysiology in many of these individuals is thought to originate from a hormonal

imbalance between the brain and the gonads. The imbalance can manifest as delayed or absent puberty, hypothalamic amenorrhea, decreased libido, sexual dysfunction and ultimately infertility. In order to design novel strategies to treat infertility and reproductive diseases, it is vital that we advance our understanding of the brain circuits underpinning normal reproductive function in the “healthy” state. We need to study how these neurons develop, map their connections throughout the brain, understand their regulation, and uncover the signals they use to communicate. The second more translatable goal of this research is to reveal changes in these circuits that underlie reproductive brain aging (e.g., from puberty to reproductive senescence) and understand how homeostatic disruptions lead to various reproductive diseases. Precisely identifying the disruptions in these hormone circuits has tremendous potential for humans. New methods could be designed to improve strategies for several reproductive impairments, such as menstrual cycle dysfunction, sexual dysfunction, and pubertal disorders.

What outputs do you think you will see at the end of this project?

The expected benefit of our project is the generation of new knowledge about the fundamental molecular mechanisms utilised by the “healthy” brain to control puberty, reproductive function and associated behaviours in mammals. In addition, we expect to generate new information about changes to neuroendocrine circuits that contribute to reproductive dysfunction to cause infertility. This output will most likely take the following forms:

*Scientific presentations and open-access research publications in peer-reviewed international journals reporting the findings of these studies.

*Datasets (e.g., epigenetic and transcriptome sequencing data, histology data, physiological data and behavioural data) published as data notes and/or deposited in public, open-access databases.

*Protocols reported in open access publications, online and/or in methods papers.

Who or what will benefit from these outputs, and how?

Our research on the ‘healthy’ neuroendocrine axis will provide essential foundational data for our disease-focused research as well as be of interest to researchers and clinicians worldwide. The primary beneficiaries in the short term will be members of the scientific community at large. Our research program, which takes a systems-level approach, is expected to yield novel data on various aspects including puberty, fertility, hormone levels (such as oestradiol and testosterone), brain circuit development (including sex differences in hormone levels, as well as systemic effects of modulating specific groups of neurons), and associated behaviours (such as circadian/daily physical activity patterns, sexual and social behaviours). These findings are anticipated to generate significant interest among researchers worldwide, extending beyond the field of reproduction.

This work aims to uncover new information about hypothalamic neurons and their regulation/molecular mechanisms. It has the potential to advance our fundamental understanding of how brain processes are altered to cause diseases of the reproductive system and whether they can be positively or negatively modulated to restore function.

The project will likely identify new pathways and factors that play a role in fertility, particularly relevant to individuals with reproductive diseases or associated behavioural

disorders (such as sexual and social disorders). In the long term, these findings could lead to novel targets for pharmacological interventions. The ability to manipulate hypothalamic hormone signalling holds immense therapeutic potential for treating diseases related to reproduction or sexual behaviours, improving IVF treatments or diagnosing patients with reproductive disorders (e.g. through a novel hypothalamic stimulation test).

While we are not currently developing hormone analogues, we expect that pharmaceutical companies will utilise our findings for their own drug discovery and development programs. Targeting treatment upstream of the GnRH neuron to restore appropriate GnRH pulsatility may be beneficial for conditions characterised by decreased luteinising hormone production, such as hypothalamic amenorrhea or delayed puberty.

Our findings will be disseminated to other scientists through peer-reviewed journal publications and presentations at scientific conferences and meetings.

Additionally, we will continue to share our advanced research methods (e.g. antibodies, probes, viral vectors, cell lines, etc) with colleagues in the UK and worldwide.

How will you look to maximise the outputs of this work?

We will maximise our research outputs by sharing our findings initially with our collaborators at scientific group meetings, then through scientific presentations at local, national and international conferences and subsequently via publication in leading peer-reviewed international journals. As standard, we publish our work in open access formats, so that it reaches the widest possible audience, including clinicians as well as non-specialist stakeholders. Timely dissemination of our research findings will enable establishment of new collaborations with a broad range of scientists across fields as well as industrial partners. Unsuccessful projects/experimental approaches will be shared via conference proceedings and with our network of collaborators when discussing best practice and designing future research methodologies or be published as stand-alone articles. We will also share our findings with the broader public via websites, social media platforms, and outreach events.

Species and numbers of animals expected to be used

- Mice: 4000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is the species of choice for these studies due to its highly characterised hypothalamic- pituitary-gonadal axis, including the availability of transgenic lines, antibodies and probes that accurately identify relevant cellular relationships. In addition, our ability to genetically alter the mouse germline is superior to that for any other mammalian (or vertebrate) species and a high degree of functional similarity exists between human and murine neural control of reproduction. These attributes make the mouse an organism of choice for accurate modelling of human physiology and diseases affecting the reproductive system.

To investigate the neuroendocrine mechanisms required for puberty and fertility, we study mice at both juvenile stages (baseline pre-pubertal stages) and adult stages (fully matured reproductive system).

This enables us to compare and understand the essential processes involved in achieving reproductive milestones or the dysfunction that leads to disease. These research questions cannot be answered without animals, as we will link neuroendocrine mechanisms to animal physiology and behaviour in living animals using viral/genetic manipulations. We are unable to utilise chordate or non-mammalian vertebrate species that are less sentient as the reproductive neuronal circuits are too different to mammals and do not have the right complement of factors. For example, the avian lineage lacks a kisspeptin gene altogether and other non-mammalian vertebrates possess different combinations of signals regulating reproduction making many comparisons difficult.

Typically, what will be done to an animal used in your project?

Most of the mice (~75% of an estimated total of up to 4000 over 5 years) will be used in our breeding and maintenance protocol to generate groups of genetically altered mice for our studies. As part of this protocol small tissue biopsies are required from animals to determine their genetic status. A subset of animals (~25%) which are born with the correct genotype will be used for experiments. As part of these experiments, some animals (~20% of all animals used on project) will receive brain infusions of non- pathogenic viral vectors (recombinant adeno-associated viruses [rAAVs]) to label, alter or monitor specific subsets of cells within the nervous system. Infusions will be performed under general anaesthesia and most animals will make a rapid unremarkable recovery.

My first research stream concerns the reproductive neuroendocrine function in the “healthy state”. The primary objective is to identify previously unknown cellular components that regulate hypothalamic neurons, which play a crucial role in controlling puberty and fertility throughout an individual’s life.

To understand the relationship between different populations of cells within the brain, we will selectivity label these cells based on the genes they express and/or use labelling techniques involving viral vectors or fluorescent dyes. This will enable us to create anatomical maps that illustrate their connectivity and understand how they change across altered physiological states. We will primarily utilise avirulent adeno-associated vectors due to their robust expression and additional advantages, such as the ability to restrict expression to specific cells. For instance, by exploiting the Cre-Lox recombination system, we can effectively label specific cells by delivering Cre-dependent viruses into the brain of genetically altered mice where specific cells express Cre recombinase (Cre-driver lines). This restricts the activation of viral transgenes exclusively within the Cre-expressing cells. In most anatomical labelling experiments, animals will need to be perfused with fixative to ensure that tissue of sufficient quality is obtained for labelling protocols.

Additionally, we can determine the molecular composition of interconnected neurons by co-labelling immunohistochemistry using antibodies against neuropeptides and neurotransmitters (e.g., vasopressin, kisspeptin, GAD67, etc.). This approach allows us to identify novel regulators of connected neurons. We will also conduct investigations into the global gene/methylation profiles from populations of hypothalamic neurons at various reproductive stages, such as during the ovarian cycle or in animals with varying levels of sex steroid feedback. The levels of sex steroids have a significant impact on cell number and gene expression within reproductive brain circuits. One objective of this research is to

analyse the gene profiles from target neuronal populations and understand how these patterns are influenced by sex steroid milieu. This will allow us to identify the functions and unique characteristics of the cells under study.

We will investigate candidate cell surface receptors and signalling molecules identified by gene sequencing to determine their ability to regulate circuit-level activity. This analysis may involve calcium imaging in live mouse brain preparations combined with manipulation of signalling pathways identified from gene expression experiments. In some instances, genetically altered animals will receive brain infusions of viral vectors to selectively express proteins, in specific cells of interest, enabling the monitoring or control of cell activity. Scientific endpoints will include identifying novel reproductive regulators, quantifying neuronal activation (e.g. c-Fos, a surrogate marker of neuronal activation) and the identification of target neurons may be confirmed by co-expression immunocytochemistry on post- fixed tissue.

Based on the identification of novel genes or neuronal populations in “healthy” reproductive brain, we will examine the specific contributions of genes or neuronal populations in controlling reproductive physiology, physical activity patterns and associated behaviours in male and female mice in vivo. To achieve this, we will employ genetically altered (GA) mice and utilise viral transduction techniques and neuronal circuit manipulations (e.g., CRISPR gene knockouts/gene over-expression) to target specific molecules in these pathways. In addition, we may experimentally alter sex hormone levels by performing gonad removal (castration/ovariectomy) to investigate if sex hormone feedback plays a significant role in regulating neuronal activity. Hormone manipulations are expected to result in only subtle effects on physical development, while effects on brain function and behaviour are predicted to be fully compatible with normal survival rates and a good quality of life (i.e. no severe outcomes are anticipated). As hypothalamic neurons express various hormones and neurotransmitters, we may also administer reproductive hormone analogues, neurotransmitters and chemogenetic drugs (e.g., DREADD actuator Salvinorin B) to challenge the endocrine system during adolescence and/or adulthood, examining how these manipulations impact the reproductive axis and behaviours. Blood samples will be collected from superficial vessels at different developmental stages or during adulthood (e.g. across the oestrous cycle) to monitor the changes to reproductive hormone levels (e.g. luteinising hormone, estradiol, progesterone). Additionally, we will conduct behavioural testing using a range of assessments to examine the impact of circuit level manipulations to physical activity patterns, sexual and social behaviours. These tests are designed to understand the neuroendocrine mechanisms underlying sedentary activity, sexual and social disorders. The minimum age for behavioural testing will be postnatal day 25, and the maximum age will be 12 months. Sexual behaviour tests will involve pairing experimental mice of opposite sexes and recording the frequency of mounts, intromissions and ejaculations as well as the latency to these behaviours. Social interaction tests will include pairing adult test animals with juvenile counterparts measuring the time spent sniffing, following, grooming and mounting over a set period (e.g. 5 min). The use of a juvenile stimulus is expected to reduce aggressive behaviour in adult mice. None of the behavioural tests are intended to induce pain, and any stress experienced is predicted to be short-lived and mild. The test will not be repeated excessively to minimise potential harm.

Throughout the project, all mice will be closely monitored for adverse reactions to surgeries, viral vectors and drug administrations. Signs of respiratory distress, ruffled fur,

weight loss and behavioural indicators of pain using the mouse grimace scale and body condition score (BCS) will be considered in assessing their well-being.

At the conclusion of the experimental paradigms, tissue will be harvested to assess histological measures, neuronal connectivity, gene expression patterns/profiles and activity.

What are the expected impacts and/or adverse effects for the animals during your project?

Some homozygous transgenic mice generated on our project may exhibit reduced fertility or sterility due to altered hormone levels. However, these effects are not expected to lead to any adverse or harmful phenotypes.

Tissue collection for genotyping through ear notching involves only slight and temporary discomfort, with no healing problems. Mild pain may be experienced by some animals during blood sampling or intraperitoneal injections. In case of bleeding after blood sampling, local pressure will be applied to stop the bleeding. The administration of substances as well as activation or deletion of genes, is not anticipated to have adverse effects.

Prior to stereotaxic surgeries, mice will be administered inhalation gases to induce general anaesthesia and a local analgesic will be applied. Stereotaxic surgery will be used to administer viral vectors or labelling agents to specific brain regions using stereotaxic coordinates. Surgical sites will be closed using tissue adhesive or sutures and incisions will be monitored for signs of inflammation. The risk of infection will be minimised by good surgical techniques and maintaining aseptic conditions.

Some animals may experience postoperative pain during recovery from anaesthetic, but they are expected to make a rapid unremarkable recovery. Perioperative analgesia will be provided and mice pain scored using Mouse Grimace Scale (MGS) to assess effectiveness. Following the surgical procedures, animals will be provided palatable analgesic jelly for alleviation of pain and their weight will be monitored to ensure a prompt recovery. Animals that lose >15% body mass will be humanely killed.

Surgical removal of the gonads and implantation of osmotic mini-pumps or silastic capsules may cause pain and local bruising, but it should not cause lasting harm. Adequate doses of analgesics will be provided, and the risk of infection will be minimised by good surgical and aseptic practices.If incisions open, the wounds without obvious infection may be re-closed on one occasion within 48 hours of initial surgery. In the event of recurrence, NVS advice will be followed.

Administration of substances will result in no more than transient discomfort and no lasting harm.

Monitoring activity, sexual and social behaviours are predicted to replicate and evoke naturalistic responses. None of the behavioural tests is designed to induce pain and any stress is predicted to be short-lived and mild.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

According to our previous project licence, we anticipate that the procedures performed on the majority of animals (approximately 75%) will fall under the sub-threshold or mild classification in terms of severity. For some animals (around 23%), it is expected that they will experience a moderate severity classification due to surgical procedures. Lastly, a smaller subset of animals (~2%) to be classified as non-recovery.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In order to study and understand the signals governing the neuroendocrine control of mammalian reproduction, which are highly complex and often involve interactions with multiple cell types in their microenvironment, and regulation by cell-cell contact (synaptic neurotransmission) and secreted factors (peptide and steroid hormones), it is necessary to study tissue from the mammalian central nervous system. We currently do not have the ability to reproduce these conditions outside an organism.

Ex vivo and in vitro assays can and will be used whenever possible to determine how subsets of neurons are modulated and function. Extracted brain tissue can maintain neuronal activation and signalling properties ex vivo for a limited period of time and in these cases, ex vivo culture/calcium imaging will be used as surrogate assays for intact neuronal function.

Which non-animal alternatives did you consider for use in this project?

For some research questions we utilise in vitro cell lines, ex vivo tissue or human post-mortem tissue in parallel with mouse tissue to understand if processes (neuroendocrine signalling mechanisms) are shared across species and models. We continue to search the literature (PubMed, Google Scholar) for non-animal alternatives for modelling the reproductive endocrine system but as stated in a recent review, “it remains challenging to predict endocrine related disease outcomes in intact animals based on non-animal test data”. One primary reason for this situation is we are still lacking knowledge of the mechanism-of-action for crucial casual elements, combined with the significant difficulty replicating the intricate multi-organ endocrine system.

Why were they not suitable?

We currently utilise a number of different neuroendocrine cell lines, such as immortalised murine kisspeptin cell lines and they are suitable for a limited number of research questions. Our studies are focused on identifying and examining upstream signaling mechanisms (unidentified circuits upstream of kisspeptin neurons) so the immortalised neurons available have limited use. Recently, we were interested in using the immortalised

kisspeptin neurons to understand whether the gene Mkrn3 (a gene critical for controlling puberty) had actions through regulating two populations of kisspeptin neurons.

We attempted to utilise these immortalised kisspeptin neurons (mentioned above) but were unable to detect any kisspeptin expression from one line, indicating that it has undergone a fundamental change and does not contain the requisite complement of factors required for our studies. We continue to use the second cell line to investigate the molecular mechanisms through which novel genes implicated in regulating puberty impact the expression of reproductive neuropeptides. However, any evolving concepts will need to be replicated and refined in an intact system (animal) where the complex environment of the hypothalamic-pituitary-gonadal-axis is present. Ex vivo and in vitro assays can and will be used whenever possible to determine how subsets of neurons are modulated and function.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 this PPL, a statistician helped us with calculations using typical variations from our own earlier experiments, results from collaborators or from publications from researchers working on similar questions to calculate the minimum numbers of animals to be used whilst ensuring the results are statistically significant. When available, sample sizes for our experiments are estimated from past experiments in combination with using the NC3R's Experimental Design Assistant. For mouse lines we breed inhouse, we used our historical breeding records to estimate the number of animals that will be required for breeding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For our quantitative experiments, we utilise the NC3R's experimental design assistant (EDA) to plan and run our experiments, practical steps, allocations, blinding and statistical analyses. We employ statistical software to estimate sample sizes and apply appropriate statistical analysis methods. We include experimental design diagrams and share these with our users and animal housing staff to support planning of and running our studies. We also reduce the number of animals required for our studies by employing multiplexed analysis combined with highly sensitive and information-rich detection techniques to maximise the amount of information extracted from each test sample.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We constantly optimise our breeding strategies to maximise the number of animals with the necessary genetic alterations. For instance in some studies we require heterozygous mice so we pair homozygous mice with wildtype littermates, resulting in only heterozygous offspring. However, in our research on fertility genes, some homozygous transgenic mice are not fertile. Therefore, we need to use heterozygous pairings, and some of the offspring

will not be suitable for our experiments. These animals may be used for future breeding or as littermate controls.

To determine the appropriate number of animals needed for valid results, we often conduct pilot studies to understand the variability in experimental outcomes. We continue to utilise high resolution technologies and integrated in vivo approaches, such as delivery of viral vectors that provide clear signals and enables us to collect multiple recordings, and samples from each subject. To minimise the number of animals required for experiments we will use state-of-the-art genetic models, including models with cell-specific expression of molecules to precisely evaluate experimental interventions and avoid off-target effects and adverse phenotypes in tissues not relevant to the analysis. An off-target effect often disturbs the wellbeing of the animal, and confounds data analysis, making additional control experiments necessary. In this manner, precise biological answers may be obtained with a minimal number of animals.

Furthermore, we actively collaborate with other researchers by sharing surplus tissue or any surplus animals that are suitable for their studies. This ensures that animals resources are used efficiently and responsibly.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the species of choice for these studies due to its highly characterised hypothalamic- pituitary-gonadal axis and the high degree of functional similarity to human neural control of reproduction. In addition, our ability to genetically alter the mouse germline is superior to that for any other mammalian (or vertebrate) species and there is an extensive availability of antibodies and probes that can accurately identify the relevant cellular relationships. These attributes make the mouse an organism of choice for accurate modelling of human physiology and those diseases affecting the reproductive system.

To minimise suffering, we have developed precise protocols to interrogate specific brain cell populations using avirulent viruses that are not known to cause any adverse health effects. Thus it is not necessary to inject more virulent strains (e.g. modified rabies virus) or utilise less specific nervous system cell ablations. After all surgeries, animals typically recover, show normal behaviour and are returned to their home cage until experimental treatment. Uncommonly, animals that fail to do so or exhibit distress or significant ill health will be killed by a Schedule 1 method unless a program of enhanced monitoring and care is instituted until the animal fully recovers; and any animal not fully recovered from the procedures within 6 hours (eating, drinking and return to normal behaviour) will be humanely killed.

Why can’t you use animals that are less sentient?

To explore and understand the intricate brain signals that control mammalian reproduction, it is crucial to study tissue from the adult mammalian central nervous system. Immature or juvenile life stages lack the developed neurocircuitry and the factors essential for fertility which include multiple cell types communicating by both cell-cell contact (e.g. synaptic neurotransmission) and circulating factors (e.g. steroid hormones).

Unfortunately, we are unable to utilise chordate or non-mammalian vertebrate species that are less sentient as the reproductive neuronal circuits are too different to mammals and do not have the same complement of factors. For example, the avian lineage lacks the kisspeptin gene altogether and other non-mammalian vertebrates possess different combinations of signals regulating reproduction. This is highlighted by my earlier publication on the reproductive circuit in the zebrafish. Zebrafish are part of the teleost lineage, which have undergone an additional whole genome duplication event. This results in teleost fishes having two paralogous GnRH genes, two kisspeptin genes, four GnRH receptors and four kisspeptin receptors. This adds immense complexity while trying to study the mechanisms governing the neural control of reproduction as multiple gene copies often results in divergent functions or redundancy. In fact, kisspeptin signalling does not appear to be essential for reproduction in zebrafish as genetic knockout mutants exhibit normal gonadal maturation, whereas in mice and humans, kisspeptin mutations lead to a state of stalled puberty and infertility.

In some studies, we intend to utilise animals that have undergone terminal anaesthesia for certain ex vivo assays. Extracted brain tissue can retain neuronal signalling properties for a limited duration. This will enable us to investigate the modulation and functioning of specific subsets of neurons as surrogate assays for modelling intact neuronal function.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The majority of animals will experience no harms associated with the genetic alterations they harbour and will be killed via humane methods prior to tissue extraction. We will minimise the number of animals that require additional procedures (infusion of substances) by seeking out and utilising non- harmful genetic approaches where possible. Where surgery is required to infuse viral vectors, we will use appropriate peri-operative care measures, as advised by the NVS, and surgeries will be carried out under aseptic conditions.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will employ the PREPARE guidelines when planning and conducting our studies. We will follow LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017). In addition, we will use the 3Rs resource library for husbandry and in vivo techniques (e.g. Grimace Scales, mouse blood sampling, etc.).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will maintain an ongoing dialogue with our skilled team of animal care technicians to explore potential improvements to our methods. Furthermore, my membership on the Animal Welfare Committee provides valuable insights as we routinely discuss advancements in the principles of the 3Rs. Additionally, we will actively seek ideas for

improvements through forums associated with organisations like LASA (Laboratory Animal Science Association) and the NC3Rs (National Centre for the Replacement, Refinement, and Reduction of Animals in Research) newsletter and resources and workshops advertised by our Home Office Liaison Officer or NVS.

80. Studying the tumour microenvironment to find better 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

Key words

Cancer, Tumour microenvironment, Metastasis, Therapy resistance

Animal types	Life stages
Mice	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To investigate the tumour microenvironment in gastrointestinal cancers to identify vulnerabilities that can be therapeutically exploited.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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-related death is the leading cause of death worldwide and better therapies are urgently needed. Emerging scientific evidence shows that the tumour microenvironment promotes cancer progression and impacts therapy response. Thus, it is important to better understand the cancer- promoting functions of the tumour microenvironment and to reveal vulnerabilities that can be therapeutically exploited.

What outputs do you think you will see at the end of this project?

The primary potential benefit relates to new scientific knowledge about cancer disease progression and to better understand why certain cancers don't respond well to current available therapies. Our studies aim to better understand how the tumour microenvironment (TME) impacts cancer progression and its response to anti-cancer therapy, with a translational focus on gastrointestinal cancers (i.e. pancreatic cancer, colorectal cancer). Our research programme has already been extensively reviewed by scientific leaders in the field. These scientific experts found that our research programme addresses an important medical need, is of high scientific quality, and that we as a team are ideally placed to carry out the proposed work. Thus, the here proposed animal project licence will allow us to continue to conduct the proposed funded work over the next five years. The aim is to get new insights into the complex molecular and cellular biology of the tumour microenvironment, the reciprocal communication between malignant cells and immune/stromal cells and immune/stromal – immune/stromal cells, and to identity vulnerabilities that can be therapeutically exploited. We aim to publish our findings in open access peer reviewed journals to advance the knowledge in the cancer field. We work closely together with our clinical colleagues allowing a rapid transfer of our new knowledge into the clinical setting. Our ultimate aim is to create impact and improve patient outcome. For example, our current project licence has already allowed us to identify a promising new target in the tumour microenvironment that promote metastatic relapse in solid cancer and together with our clinical colleagues and the support from our local charity and national charity we now aim to move these findings into clinics.

Thus, outputs from the here proposed project licence include publications of our findings in peer reviewed open access academic journals to advance the knowledge in the cancer field and ultimately to translate our findings into clinical application.

Who or what will benefit from these outputs, and how?

This research will benefit academic and clinical researchers across basic and applied cancer research, in addition to pharmaceutical sectors interested in developing better cancer treatments. The ultimate long-term beneficiaries will be cancer patients and their families, patient support groups and healthcare professionals delivering medical care to cancer patients.

How will you look to maximise the outputs of this work?

We will publish our data in open access, peer-reviewed journals and we will deposit our data in open source databases that will facilitate their dissemination within the scientific community and allow colleagues and their research programs to benefit from these studies.

Scientific presentations and open access peer reviewed publications:

We will publish our data in open access, peer-reviewed journals and we will deposit our data in open source databases that will facilitate their dissemination within the scientific community and allow colleagues and their research programs to benefit from these studies.

Scientific presentations and open access peer reviewed publications:

As the main output of this research will be scientific, the primary method by which we will disseminate the results of this research will be through open access, peer-reviewed publications. Progress in the research project will also be communicated through national and international scientific meetings and conferences, many of which are attended by patient groups, clinicians and healthcare workers, thus giving us the opportunity to engage these groups in our research activities. Within our institution, our research groupings hold weekly research in progress meetings where data is presented and discussed, allowing the opportunity to get feedback, advice and support from colleagues. We also regularly host external speakers for institutional seminars and discussions.

Digital Newsletters & institutes Websites:

We will engage with our in-house Public Relations office who will take responsibilities for communicating our work to the public, often in the form of press releases and publishing news stories on our institution's website. The results from our research studies will be communicated to all researchers at our institution through our weekly Institute newsletter and through our laboratory websites.

Communication with Patients and fundraisers:

Our group are actively involved in the local Clinical Trial Unit-led Patient and Public Involvement (PPI) programme in our region, promoting the need to understand, prevent and treat solid cancers (including pancreatic cancer). The PPI group enables patients and the public to learn about biomedical research carried out at the university, which ultimately will provide benefits to patients in our region. Together with a Senior Research Nurse (head of the PPI), we organize an annual evening for cancer patients and their relatives as well as lab tours. During this event, we discuss the molecular drivers of cancer, the interaction between immune/stromal cells and cancer cells, and general on-going research efforts undertaken to fight solid cancers, including our own research.

Public engagement and outreach activities:

Researchers involved in this project plan to further participate in public engagement & outreach activities, including lab tours for the public & students, evenings organised by the local Clinical Trial unit Patient and Public engagement team for cancer patients to promote the need to understand, prevent & treat cancer, and workshops on the "Human Body" organised for local schools.

Species and numbers of animals expected to be used

- Mice: 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.

Living animals are required because the complexity of the process being studied cannot be duplicated or modelled in simpler systems. In addition, preclinical studies in living animals are required by federal regulations prior to human testing. We will perform the

experiments in mouse models since mice are phylogenetically lowest species that provides adequate size, tissue and anatomy for the proposed studies. In addition, previous studies using mouse models formed the background of the studies proposed here and large databases exist for mouse studies allowing comparison with previous data.

Our work focuses on a range of solid cancer types occurring in adult humans. The older we are, the more likely we are to develop cancer. Most cases of cancer are in people aged 50 and over. It is now well documented that organs, tissues and cells change their functions during the life time and thus respond differently to stimuli from their surroundings. Hence, we will use adult mice at different life stages, including aged mice (~12 months), to reflect the human diseases.

Typically, what will be done to an animal used in your project?

Animals might undergo one or more of the following procedures:

A) Surgical procedures: For the implantation of cancer cells into the pancreas, cecum, spleen or portal vein, we will perform 10-20 min surgeries per mouse, where we will typically make a very small incision into the abdomen of the mouse, implant with a specialised syringe cancer cells and then closing the wound with suture. Surgical procedures will also be used to resect established tumours, using a similar procedure as applied for cancer patients. Briefly, a very small incision into the abdomen of mice will be made, followed by surgical resection of the tumour mass and then closing the wound with suture. All surgical procedures will be carried out under anaesthesia. All animals will receive analgesia perioperatively to reduce surgery related pain. Animals will only be aware of the anaesthetic being administered and may experience mild distress and no pain.

B) Substance administration and collection of blood samples: Typically, animals will experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal) or oral gavage. Where administration is required for prolonged periods, animals will be surgically implanted with slow release devices such as a mini-pump or substances will be added to the drinking water. For the use of mini-pumps, these animals will experience some discomfort after surgery and some mild to moderate pain which will be treated with analgesics. Animals will undergo changes in diet which are not expected to cause distress but may sometimes result in obesity or itchy skin. Some diets may result in weight loss due to unpalatability. Animals will be placed onto normal diet should they lose 20% of their body weight. Animals will experience mild and transient discomfort from blood sampling. 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.

C) Spontaneous pancreatic tumour model: Our programme of work on pancreatic cancer also includes the analysis of tumour development using a mouse line harbouring genetic mutations known to be responsible for pancreatic cancer development. In this model, the mice develop spontaneous invasive pancreatic tumours, starting around week 6. Metastatic spreading to the liver occurs unpredictably during disease progression (between 10-30 weeks). Mice bearing primary tumours of 4-6 mm will be transferred into the study and treated with standard of care chemotherapy (every 3rd day) or vehicle control, and monitored regularly (2-3 times/week). These mice recapitulate well the pathophysiological characteristics of human pancreatic cancer disease progression and

are largely refractory to chemotherapy treatment. Hence, we anticipate humane endpoint within 2-3 weeks after transfer into the study, independent of treatment group. Human endpoint will be within moderate severity. Mice with advanced pancreatic cancer disease might experience mild distress and mild pain due to the presence of cancer.

D) Experimental measurements/in vivo imaging: Our main focus is to quantify tumour burden and how tumours respond to treatments. Tumour formation will be induced by implantation of tumour cells or by using a genetic engineered mouse model (GEMM) of pancreatic cancer. We have established reliable in vivo imaging techniques, including high frequency ultra sound, bioluminescence imaging, and MRI to quantify tumour and/or metastatic burden in vivo during the course of the experiments. In vivo imaging will be conducted under anaesthesia and animals will only be aware of the anaesthetic being administered and may experience mild distress but no pain.

What are the expected impacts and/or adverse effects for the animals during your project?

The main adverse effects for our studies will be caused by the presence of cancer disease progression. During tumour development, animals might experience pain, weight loss, loss of appetite, change in body condition, failure to respond to gentle stimuli, and laboured respiration. These symptoms appear toward the end-stage of cancer development with an estimated duration of 5-7 days. We will humanely terminate the mice prior they progress to severe adverse effects.

Several of our protocols involved internal tumours, which are challenging to monitor. Thus, for all internal tumour studies, we will first conduct pilot studies to refine minimal cell number needed for injections and to determine impact of tumour burden on animal health.

In subcutaneous tumour models, ulceration might occur. Mice with ulceration will be humanely terminated.

Where surgery is required, animals might also experience temporally minor pain during the recovery of surgery, with an estimate duration of 2-3 days.

Cancer patients can undergo multiple surgical interventions to remove tumour mass with a curative intention. Thus, in our programme of work, some of the animals will also have up to two surgeries to remove tumour mass. Each surgical procedure has a curative intention and only animals who have fully recovered from the first surgery will undergo a second surgical procedure. Animals who don’t fully recover from surgical procedures will be humanely terminated.

In some studies, we will use aged mice (~12 months). In aged mice, additional expected adverse effects are weight loss, poor coat , poor coat conditions and/or hair loss, abnormal and/or unsteady gait, dermatitis, cataracts, signs of ascites/oedema. We have a good track record in our facility for monitoring aged mice as we have done this for many years.

Expected severity categories and the 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: 40% mild, 60% 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?

Living animals are required because the complexity of the process being studied cannot be duplicated or modelled in simpler systems. In addition, preclinical studies in living animals are required by federal regulations prior to human testing. We will perform the experiments in mouse models since mice are phylogenetically lowest species that provides adequate size, tissue and anatomy for the proposed studies.

Specifically, our studies aim to decipher the cross-talk between malignant cancer cells - non-malignant stromal cells, and stromal cells - stromal cells in solid cancers. While analysing cell-cell interaction in vitro using isolated 2D and 3D co-culture models can be very informative, they do not precisely reproduce cell-cell communication in vivo. This is because the surrounding microenvironment, including neighbouring cells and extracellular matrix, cytokine and growth factor milieu profoundly influences their interaction and subsequently their biological functions. The complexity of the tumour microenvironment (TME) cannot currently be accurately reproduced in vitro. Taken together, in vitro assays cannot adequately model the complexity of the TME in cancer. Therefore, further in vivo work is required.

Which non-animal alternatives did you consider for use in this project?

Where possible, we will use alternative non-animal models to study cell-cell interactions in vitro. We have established a variety of complex 2D and 3D cell co-culture assays and we will harness of these new methods to study mechanistic actions of cancer promoting factors. For examples, we can test the effect of identified cancer promoting factors on immune cell activation in vitro. We shall maximise this latter approach as much as possible. We have also established colony formation assay where we can study how the presence of stroma cells affect growth and therapy response of malignant cancer cells.

Finally, we have established strong interactions with our clinical colleagues allowing us to access patient material from cancers we are investigating. We are now using precision cut tumour slice models which allows us to culture for a short period of time human tumour samples and we can analyse the cross-talk of stroma cells with malignant cells.

Why were they not suitable?

Our study focuses on stroma – malignant cancer cell interactions. Stroma cells, particularly immune cells, are mainly recruited to the site of tumour formation through the blood circulation. Hence, this cannot be mimicked ex vivo and animals' studies remain required to reveal tumour promoting functions of stroma cells. Emerging evidence also shows that

cancer is a systemic disease, which can change the function of distant organs. There is evidence of organ -organ crosstalk in cancer. This currently can't be recapitulated in vitro.

Our studies have a focus on the immune system, and how cells from the immune system affect cancer disease progression. The immune response is regulated systemically, and we can't recapitulate this ex vivo.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number is based on our previous animal return and usage. We are currently using ~800 mice/annum. We aim to reduce this number to 700 mice/annum over the next five years, totalling in n=3500 animals needed.

A statistician helped us with calculations using typical variations from our own earlier experimentation to calculate minimum numbers of animals to be used whilst ensuring that the results are statistically significant. Sample sizes for our experiments are estimated from past experiments. Calculations typically show that we need group sizes of 8 to achieve the quality of results we need. We've used our annual return of procedures data to estimate the number of animals that we will need to use for breeding.

Although we have established protocols for similar experiments during the last 18 years, we plan to perform pilot experiments involving small numbers of animals where needed to account for changes in disease progression and variability in experimental groups due to changes of treatment regimens/infrastructure/material compared to our previous work. Variation observed in the pilot studies will be used for power analysis to determine sample sizes in the main experiment.

To reduce overall animal numbers, we will apply in vivo imaging techniques (MRI, PET/SPEC/CT, HFU, Bioluminescence). These techniques will markedly reduce the number of animals needed for time course studies since we will be able to accurately monitor in each individual mouse changes in tumour burden over time.

For each and every experiment, as part of good laboratory practice, we will write an experimental protocol which includes:

- a statement of the objective(s)
- a description of the experiment, covering such matters as the experimental treatments, the size of the experiment (number of groups, number of animals/group) and experimental material.
- notes of any unexpected events, including loss of animals and the reason for their loss.

- an outline of the method for analysis (which may include a sketch of the analysis of a variance, an indication of the tabular form in which the results will be shown, and some account of the tests of significance to be made and the treatment differences that are to be anticipated).

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?

At the end of the experiment, we will harvest as many tissues as possible at post-mortem. If we don’t need to analyse the tissues immediately, we will freeze them and make them available to other researchers working on similar questions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

To study cancer disease development, we will induce tumour formation by implantation of tumour cells. It is now well known, that the tumour microenvironment varies between different organs and cancer types. Hence for some studies, implantation of tumour cells will require a small incision into the abdomen to access the organs, including pancreas, cecum, liver. All surgical procedures will be carried out under anaesthesia.

To study specific steps of cancer cell dissemination, we will also employ experimental metastasis models. In this case, cancer cells will be directly injected into the blood stream or the spleen. From the latter, cancer cells are drained rapidly into the portal vein where they form liver metastasis. In experimental metastasis models, the dissemination of cancer cells is highly controllable and it will allow us to study early dissemination steps and the early outgrowth of cancer cells. To induce gene expression in animals or to deplete specific cells, some animals might be given substances by mouth, injection, or through food. Oral gavage or injection can be necessary to induce a rapid change in gene expression. This will let us to study processes that happen within short time periods of a few hours. We will also study on a molecular and cellular level how early and established tumours respond to current anti-cancer treatment therapies. Animals will be treated with therapy regimens similar used in human settings, e.g. injection of substances into the peritoneal cavity, by intravenous injections, or oral gavage.

Data from previous and future experiments will be analysed in order to use the minimum dose of substances and treatment required to exert biological effects, rather than doses that induce significant toxicity. In addition, where possible and certainly for all pilot experiments, we will analyse time points when there are early signs of disease but no late stage clinical manifestation (e.g. we will analyse cellular compositions of tumours shortly after their establishment rather than after organ failure or metastatic spreading has occurred).

Whenever necessary we shall use local and general anaesthesia to minimise animal suffering. In all tumour studies, we shall kill the mice if the mean diameter of the tumour reaches 1.2 cm determined by calibration or in vivo imaging techniques. In long term experiments, animals will be killed if they lose more than 20% body weight or if they show sign of advanced cancer disease progression such as ascites or other sign of ill health, such as hunching, lack of group behaviour or breathing difficulties. In addition, we will use body scoring system to complement cancer weight loss during disease progression to classify the health status of our animals and animals with signs of significant ill health will be terminated immediately.

Why can’t you use animals that are less sentient?

Non-mammalian animals are limited in their use because they do not develop a TME that physiologically represents the human setting. Cells from our immune system are the key drives in orchestrating the formation of a TME. Hence, non-mammalian animals 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. We can’t use embryos or very young animals as their immune system is immature and doesn’t respond to tumour formation in the way mature animals do.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For all our tumour studies, animals will be carefully monitored by staff trained to work with tumour bearing mice. In advanced disease settings, score sheets will be used to record weight gain/loss and development of adverse effects. Animals that display any sign of significant ill health will be killed humanly killed.

All surgery will be conducted using aseptic technique according to published guidance for good practice for aspetic surgery (NC3R and LASA). Consequently, post-operative infections are not expected. We have more than 18 years experience in conducting small animal surgeries and we have several trainee surgeons in our team. Hence, we have optimised our surgical procedures following surgical practice used in patients. Hence, our surgical procedures are causing minimal harm to animals. Animals will be closely monitored post-operative and in the unexpected case animals do not fully recover from surgery, animals will be referred to our veterinarian or humanly killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For the planning and design of our animals’ studies, we will follow the most recent published PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines, which also provides useful checklists that can be adapted to the individual studies. PREPARE guidelines are available here:

http://journals.sagepub.com/doi/full/10.1177/0023677217724823

Other useful online resources, including ARRIVE guidelines (available including guidance and publications from the NC3Rs and Laboratory Animal Science Association) will be used to assure a rigorous and reliable design of our in vivo experiments.

https://arriveguidelines.org/arrive-guidelines

For adminstration of substances: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3189662/

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. In addition, we will attend local and national workshops/meetings organised by NC3R and we will continuously implement new advances into our practice.

81. Investigating hormone secretion and action in metabolic 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

Metabolism, Pancreas, Diabetes, Energy Homeostasis, Regeneration

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?

Changes in the release of hormones from the pancreas, and response to the body to these hormones, are key features of so called 'metabolic diseases' such as obesity and diabetes. This project will investigate the mechanisms controlling the release and action of pancreatic hormones, how these become dysfunctional in metabolic disease, and to develop strategies to restore regulated hormone release/action.
Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Globally there are 537 million people living with diabetes, with these numbers projected to rise significantly over the coming years. This reduces quality of life, places a substantial

burden on health care systems, whilst increasing risk of mortality. There is a compelling medical, ethical and economic need to investigate the control of metabolism and nutrient balance during health, how this becomes defective during diabetes, and to inform future efforts to better manage, treat and prevent metabolic disease. Release of hormones, in particular insulin and glucagon from pancreatic islets, plays a central role in controlling safe levels of nutrients, with defects in this system underpinning both type 1 and type 2 diabetes. It is therefore a key research priority under this project to investigate the mechanisms controlling pancreatic hormone release and action, and to develop new approaches to restore regulated hormone release and action during metabolic disease.

What outputs do you think you will see at the end of this project?

The key knowledge gained from this project will be increased understanding of the regulation of hormone release and action in the context of nutrient homeostasis (regulation of nutrient levels in a safe range), and new insights into potential therapeutic strategies to restore pancreatic hormone release (in particular insulin) during diabetes. The outputs communicating this knowledge will include scientific publications, presentations and lay/public engagement talks. Intellectual property generated by this project may also be used to develop future therapies for diabetes.

Who or what will benefit from these outputs, and how?

There are many stakeholders who will benefit from this research. In the near term, the scientific community and funding bodies will benefit from new insights into nutrient homeostasis and dysregulation in metabolic disease. In the longer term, people living with diabetes, pharmaceutical and biotech companies, and ultimately the wider health care system and economy, will benefit from new insights, and potentially new therapeutic strategies to tackle dysregulated hormone secretion.

How will you look to maximise the outputs of this work?

We will communicate the research process in presentations and publications, to enable other researchers to learn from both the technical and scientific aspects of this project. The project objectives will be lead by the license applicant and host institution, but are underpinned by strong collaborative networks helping to increase the impact of this work. Our funders are active in promoting the projects, further enhancing dissemination of findings and insights.

Species and numbers of animals expected to be used

- Mice: 6300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 essential that our research project is carried out using mice, as there is simply no alternative. Glucose homeostasis and hormone secretion, action, stability and clearance

are regulated by a myriad of circulating factors (each with their own pattern of systemic regulation) and the contributions of multiple organ systems, which cannot possibly be recreated in vitro: therefore, these processes must be investigated in a living mammal to ensure our results are physiologically relevant. It is neither practical nor ethical to conduct these interventional experiments in human volunteers. We have opted to study adult mice due to 1) they are a good model of human metabolism and endocrine function; 2) there is a wealth of existing data on mouse physiology; 3) there are a large number of genetic tools available; 4) mice breed readily and quickly. Further details under 'refinements' below. Notwithstanding this, we will always replace in vivo experiments with in vitro experiments where possible, such as testing expression or basic function of a gene/protein, or optimising drug dosage. These data will then inform the subsequent in vivo testing which will establish physiological relevance.

Typically, what will be done to an animal used in your project?

Our mouse models and techniques have been selected as they are: (i) relevant to understanding human metabolic disease (e.g. diabetes, obesity etc.); (ii) can be used to address basic biological questions about the normal regulation of whole body metabolism, nutrient levels such as blood sugar (glucose), body weight and hormone secretion; (iii) have the least impact on the animal's welfare, compatible with our scientific objectives.

Typically, mice will be bred under our breeding protocols, either being a genetically altered model (e.g. to investigate the role of a candidate gene) or a disease model (e.g., a mouse model of spontaneous type 1 diabetes). Young adult mice will then be transferred to our phenotyping protocol. In some instances, young mice will be procured from other regulated project licence holders or from approved commercial suppliers.

During phenotyping, the goal will be to answer the scientific question/objective using the appropriate number of mice whilst minimising any harms. Typically, mice will have body weights and blood glucose measured weekly (via a small blood sample being taken from the tail, similar to a human finger prick test) to determine if the mice are metabolically healthy or show signs of prediabetes or diabetes. Some mice will also undergo glucose tolerance testing (or similar challenges) to determine their ability to clear blood glucose (again similar to GTTs in people), to test the effect of (for example) increasing or decreasing expression of a candidate gene. Some mice may also have a 'tracer' put in their drinking water or food: these are special benign molecules that do not alter the welfare of mouse (and many are used in humans as well) and enable us to track metabolism in specific tissues. Some mice may also be placed on a diet high in saturated fat and sugar to increase body fat and may undergo an MRI scan (similar to human studies) to measure fat mass, enabling us to test if changing a given gene alters this process. Occasionally mice will need to undergo surgery, for example to implant an insulin release pellet in models of diabetes if blood glucose becomes too high. A very small subset of mice will undergo a technique called a 'glucose clamp', which will involve surgical implantation of catheters which are subsequently used to control blood glucose and insulin levels: this technique is the gold standard for assessing insulin action. At the end of all studies will end with the mice being humanely killed by approved procedures and tissue collected for analysis and long terms storage, providing an important resource for future research.

What are the expected impacts and/or adverse effects for the animals during your project?

Our protocols have been carefully planned to minimise any harms or adverse effects to the mice being studied, both for ethical and scientific reasons, as stress/discomfort can affect the study of metabolism. Some expected adverse effects are listed here:

- Hyperglycaemia (high blood sugar) in mouse models of diabetes. This is unavoidable with these models, however we will regularly monitor the welfare, blood sugar and body weight of mice, and treat with insulin (to reduce sugar levels) when required.

- Surgical complications (<5% of mice undergoing surgery) could include reopening of the surgical site or irritation. This will be minimised by good surgical technique by competent researchers, appropriate pain relief and regular monitoring of mice. If this does occur advice will be sought from the vet and if needed the mouse culled.

- Irritation from multiple injections during drug treatment (<5% of mice being treated). Minimised by rotating the site of injection, and only giving daily injections for up to 14 days, along with close observation of mice. If this does occur advice will be sought from the vet and if needed the mouse culled.

- Hypoglycaemia (very low blood sugar) following insulin injection (<5% of insulin injected mice).

Mice will have blood sugar and behaviour closely monitored after insulin injection: signs of hypoglycaemia will trigger a sugar injection and provision of food to raise blood sugar.

Advice from the Named Veterinary Surgeon or the Named Animal Care and Welfare officer (NACWO) will be sought if any expected adverse effects are persistent, or if any unexpected adverse effects occur.

The mouse will be humanely culled if:

Weight loss of greater than 15% starting body weight.
Piloerect coat ('spiky hair', a sign of discomfort) showing no improvement after 24 hours.

Lethargy showing no improvement after 24 hours.
Persistent blood glucose level of greater than 33.3mM

A reduction in body condition, a standard scoring system used to detect welfare concerns:

Expected severity categories and the 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 two-thirds of mice studied under this licence will experience 'mild severity' meaning that any discomfort is mild and transient (for example an injection). The remaining third will experience 'moderate severity', for example due to the presence of diabetes/high blood sugar levels, or due to the need for recovery surgery (including appropriate general anaesthesia and pain relief).

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 have opted to study mice as: 1) they are a good model of human metabolism and endocrine function; 2) there is a wealth of existing data on mouse physiology; 3) there are a large number of genetic tools available; 4) mice breed readily and quickly.

It is essential that our research project is carried out using mice, as there is simply no alternative. Assessment of the regulation of hormone release, action, stability and clearance must be investigated in a living mammal to ensure our results are physiologically relevant: nutrient regulation (and hormone release action, stability and clearance) are regulated by a myriad of circulating factors (each with their own pattern of systemic regulation) and the contributions of multiple organ systems, which cannot possibly be recreated using cells in a dish. It is neither practical nor ethical to conduct these interventional experiments in human volunteers. Therefore, we have opted to study mice.

Notwithstanding this, we will always replace mouse experiments with cell experiments where possible, such as testing expression or basic function of a gene/protein, or optimising drug dosage. These data will then inform the subsequent mouse experiments which will establish physiological relevance. In addition to assessing nutrient regulation and metabolism in mice, we will also study primary cells isolated from mouse models after being humanely culled: primary cells are a far better model of hormone release or action than cell lines that are maintained purely in the lab, as they are well differentiated and have developed in response to all of the normal cues (e.g. circulating factors, nervous system, circadian rhythms) provided in the body, prior to isolation.

Which non-animal alternatives did you consider for use in this project?

This research will use mice, as there is simply no alternative. Investigating the physiological regulation of hormone release and action can currently only be done reliably in a living organism, ideally a mammal to recreate the factors that an endocrine cell would normally respond to.

However, where possible we undertake basic work (not requiring a physiological system) using cells in a dish (in vitro). As an alternative to primary mouse cells we have tested:

- transformed cancer cell lines.
- Primary human cells.
- Stem cell derived cells.

Although these are of some use to our research programme for basic cell-specific questions, they cannot completely replace the investigation of hormone secretion and action using mouse models and primary cells. For our current funded projects approximately 40% of our work will be conducted in vitro using transformed cell lines and stem cell models; 30% ex vivo using primary mouse and human cells; and 30% studying mouse models in vivo. Therefore, we are actively replacing mouse work with cell models and only move to in vivo studies when necessary and scientifically justified.

Why were they not suitable?

transformed cancer cell lines. Where possible we use, and will continue to use, transformed cancer cells lines for this purpose: however, there are profound limitations to these models as they rapidly divide and are quite different to mature differentiated primary cells, both in terms of function and behaviour. Furthermore, they do not provide the wider physiological context (systemic factors; other tissues) or developmental pathways that occur in the mouse. Therefore, we can only use these cells for very specific experiments.

Primary human cells. We will utilise primary human cells for some experiments: however, these are highly variable (preparation to preparation; donor to donor), sporadic in supply, expensive, often of low/variable quality with poor function, have to travel long distances and have their own ethical constraints. Therefore, we only use these in a limited capacity, mainly to validate findings generated in cells and mice to test translatability.

Stem cell derived cells. We have recently attempted to set up cell models derived from stem cell lines in the lab and are continuing to work on this model: at present we haven't achieved a cell that fully recapitulates primary cell function. Even the best models in the literature are no match for fully functional primary cells extracted from mice.

Therefore, at present, for the aims described in this project license application, there is simply not a viable substitute for mouse models and primary mouse cells, which provide a more real-life test compared to cell lines that have only ever been grown in the laboratory.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

To achieve our aims, we have carefully planned our experiments based on our extensive prior experience studying metabolism using mouse models, banks of metabolic data from prior studies, and technical expertise. For the vast majority of techniques, we have established protocols and expected baselines enabling us to plan to study the appropriate number of mice to test a given hypothesis/intervention to provide relevant and valid data (that will robustly address the research question/aim), and to ensure that we do not use more mice than is necessary. For the minority of techniques and models for which we

either not have extensive data sets, or we are aware of potential variability, we will make an initial plan based on available data with further refinement after a pilot study.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have carefully planned experiments to achieve the stated scientific aims using the smallest number mice. This is based on prior metabolic data generated using similar techniques and models, published data, best practice, statistical planning and tools such as the Experimental Design Assistant (EDA) produced by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs). We also use the literature and other data sources to inform experimental design, for example pharmacokinetic and bioavailability data available for small molecules. For commonly used techniques we have established power calculations and experimental design principles that can be adapted to each experimental aim. For the minority of techniques for which we do not have prior data sets, we will perform initial power calculations based on published data, with further refinement after a pilot study. Experiments will be designed following the NC3Rs 'Animal Research: Reporting of In Vivo Experiments' (ARRIVE) guidelines where possible to minimise variability and confounding factors, and to ensure experimental rigour. Our studies will use the most appropriate and rigorous controls available, to ensure that our data are valid so that study mice are not wasted. For example, study cohorts will consist of test mice and littermate control mice, to account for variations in genotype and environment.

In summary, these measures will ensure that we will study the appropriate number of mice to test a given hypothesis/intervention to provide relevant and valid data, and to ensure that we do not use more mice than is necessary.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Technical competency. We will ensure that all researchers conducting the experiments have the necessary technical competency to carry out the procedure/protocol/technique. We will use our experience to ensure that our experiments are carried out with high technical precision to avoid wasting mice due to technical/experimental problems. Technical accuracy will reduce “noise” in the data, meaning that less mice are required to achieve sufficient statistical power to test a given hypothesis.

Our breeding strategies will be designed and tailored for each mouse line to provide the most appropriate genotypes, and littermate controls, to conduct the experiments efficiently and rigorously. Where possible we will initially study both male and female mice during our experiments, thereby reducing wastage of mice. If we find a sexually dimorphic phenotype (i.e., that affects only one sex), then we may need to study just one sex.

Tissue collection and data sharing: at the end of each study, mice will be killed by approved humane methods. In addition to collecting the target tissues that will be studied immediately as part of the project, we will bank down other tissues that may be of interest at a later date. This will ensure that some future studies can be carried out, or interesting/unexpected findings followed up, without breeding and studying additional mice. Sharing of samples, resources, models and data: Data and key findings will be distributed as quickly as possible (e.g., by publication, presentation, patent and social media) to get

the results out to the academic, industrial and lay communities to inform other studies and reduce unnecessary replication of similar mouse studies. Samples, resources, models and expertise will be shared to ensure that other studies are conducted as efficiently as possible (e.g., technical competency, statistical powering etc.), thereby further reducing numbers of mice used in research. Finally, banked tissues/samples will be made available to other research groups on a collaborative basis, to enable them to utilise our models without having to breed additional live mice.

Experimental design can be refined once pilot data are available.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.We have opted to study mice as: 1) they are a good model of human metabolism and endocrine function; 2) there is a wealth of existing data on mouse physiology; 3) there are a large number of genetic tools available; 4) mice breed readily and quickly.

Our selected mouse models will enable us to: (i) understand human metabolic diseases (e.g. diabetes and obesity) that display common phenotypic and pathological features; (ii) address basic biological questions about the normal regulation of whole-body metabolism, nutrient regulation, body weight and hormone secretion; (iii) confer the least impact on each animal's welfare, compatible with our scientific objectives. For example, in some cases we may turn on (or off) gene expression in adult life (rather than from birth) to reduce the length of time that the mouse is living with the phenotype of the gene deletion (e.g., obesity or diabetes). Where possible we will induce/knockout gene expression in specific tissues rather than globally - for example, when studying a beta cell gene that also plays a role in other organs. However, we may sometimes need to activate or inactivate a gene constitutively in all tissues to more closely mimic the effect of the human phenotype, or to explore interactions between different tissues or chart developmental effects.

Why can’t you use animals that are less sentient?

We need to use adult mice (as opposed to more immature life stages i.e., embryos) as our project aims are to understand mature/adult cell function.

We cannot use other model species that may be perceived as less sentient as 1) mice are a well validated model of metabolism; 2) the genetic tools and models of metabolic disease are well established in mice; 3) there are many examples of translation of therapeutic approaches from preclinical mouse model to humans, underscoring the relevance of the mouse for studying human endocrinology and metabolism; 4) our protocols to assess metabolism are well established in mice, and experiments can be conducted in a refined manner with the least number of animals required.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Current protocols have been refined over a number of years, with some examples provided below. Notwithstanding this, we will continue to refine procedures throughout the project based on 1) developments in best practice; 2) arising data on efficacy, variability, adverse effects etc; 3) feedback and advance from research, animal welfare and veterinary staff.

These protocols for mouse phenotyping have been refined over many years, ensuring that metabolic phenotyping will be conducted with the least disturbance to the mouse whilst gaining suitably robust data to answer our research question. If a particular test has not been performed previously by a researcher/technician, or if refresher training is sought, then training will be provided by a more experienced researcher/technician. It is important to state that stress or pain will impact on metabolism and therefore confound our experimental data sets: therefore, there is a strong scientific as well as ethical rationale for us to minimise stress. The majority of our phenotyping tests are of mild severity (e.g., glucose tolerance testing), as they involve low volume blood sampling from the tail and sometimes fasting and/or a single injection.

Some refinements that have been made to our protocols:

During insulin administration, mice will only be fasted for up to 6 hours so that blood glucose remains in the fed range, thereby providing a buffer to reduce the risk of hypoglycaemia. Sex-specific doses of insulin have been established in previous studies and, although unexpected, if signs of hypoglycaemia occur then a bolus of glucose will be given by injection and the mouse removed from the procedure and allowed to recover with close monitoring.

Fasting mice for over 6 hours for any test procedure will be conducted no more than once per week. Only one tolerance test may be performed in the same week.
Models of diabetes (Db/Db, NOD, STZ-induced) will not be kept past 30 weeks of age.

Where applicable, the use of tissue-specific genetically altered mice enables deletion of a given gene in the tissue of interest, which is a refinement over the use of global knock-out mice which generally have a stronger phenotype.

Tamoxifen administration (to induce transgene activation) will typically be administered by oral gavage, as subcutaneous injection in corn oil can cause a hardening of the skin. We will also explore the use of voluntary ingestion of tamoxifen to remove the need for gavage.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We aim to conduct experiments in line with the NC3Rs 'Animal Research: Reporting of In Vivo Experiments' (ARRIVE) guidelines and using best practice within the research field.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will monitor updates from the NC3Rs and from animal welfare staff regarding best practice in experimental design, technical skills, husbandry and handling. We will regularly attend user meetings to keep up to date with developments.

82. Sensory and emotional processing in 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

Pain, Stress, Depression, Environment

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant
Rats	neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our goal is to achieve a deeper understanding of the neurobiology of pain states in order to offer better pain relief. In particular, we want to investigate the intricate relationship between stress/anxiety and pain experience and explore how the sensory and affective component of pain can influence each other.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chronic pain affects at least 20-30% of the human population, reducing patients' quality of life and ability to work, therefore presenting a huge social and economic burden. Chronic pain can be a consequence of accidents, surgery, diseases such as diabetic peripheral neuropathy or drug treatments like chemotherapy, and up to 16% of Europeans suffer from

moderate to severe pain that does not resolve even though the injury has healed. Less than 50% of chronic pain patients achieve pain relief and this relief is often only partial and is accompanied by unpleasant side effects. Moreover, depression and anxiety are co-morbid symptoms in many chronic pain patients. We work on the assumption that if we understand the underlying molecular changes in pathways that are activated by painful stimuli, new treatments that will improve not only chronic pain but also co-morbid affective conditions will be generated, therefore leading to a significant improvement of pain patients' quality of life.

What outputs do you think you will see at the end of this project?

The outcomes of our research will be published in open access journals and presented at national and international congresses attended by basic scientists and clinicians.

Who or what will benefit from these outputs, and how?

Our work will undoubtedly provide benefits to a number of people.

- Our group first of all will use the data produced with this project to obtain further funding for research.

- Other researchers in the field will also rapidly directly benefit from the advancement of the understanding of the neurobiology of pain states that our work will provide.

- Pharmaceutical companies and NIH have already shown their interest of our work and are constantly on the lookout for new potential targets for the treatment of persistent pain states. Our work is of substantial translational value and of particular importance in the current battle against addiction and drug overdose that has been triggered by over-prescription of opioids for chronic pain in USA.

- In the longer terms, clinicians and patients will also benefit from our work, in particular in clinical settings where patients are constantly looking to understand their condition and questioning how proposed treatments work. Chronic pain patients will also directly benefit from the identification of novel targets when new drugs are available for clinical use which ultimately will reduce the financial burden of chronic pain on the society.

How will you look to maximise the outputs of this work?

- We always maximise the output of our work as follows:

- We publish all the data with acquire, regardless of the outcome of the experiments (positive or negative).

- We participate to national and international meetings to share of work but also to ensure continual career development and to update our knowledge on best practice.

- We collaborate with academic specialists from other fields when we are not the best suited persons to run a type of experiments.

- We outsource some of our work to obtain the best outcome possible and spend our time where our skill sets is best suited. This is particularly important when working with valuable animal tissue.

Species and numbers of animals expected to be used

- Mice: 3000
- Rats: 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 will use rats and mice because information gained from experiments in these species quite accurately reflects many aspects of pain processing in humans. We will use both adult rodents to ask about pain mechanisms in adulthood but also young rodents to investigate pain experience in younger animals. We will also look at the impact of early life adversity using stress models in very young animals (from post-natal day 2).

Typically, what will be done to an animal used in your project?

In this project, the behaviour of animals will typically be observed before and after the development of an hypersensitive state that will be induced by chemical (e.g. injection of an inflammatory agent) or, less often, surgical means (e.g. ligation of the sciatic nerve to induce neuropathic pain) but also simply exposure to fat diet or stressful events. For a subset of animals, we will investigate whether exposure to stress, prior or after the initiation of the pain state, can exacerbate the pain experience. One of our aim is to compare stress of different origins (e.g. maternal separation or deprivation, restraint stress and forced swimming). These different stressors are likely to provoke different profiles of biological responses and we want to identify stressors in rodents that could resemble stress responses seen in humans that may promote susceptibility to chronic pain. Finally, we will try pharmacological and non- pharmacological means (e.g. exercise, environmental enrichment) to improve the pain state that may have been exacerbated by exposure to stress.

What are the expected impacts and/or adverse effects for the animals during your project?

During our project, our animals will indeed be exposed to pain, as defined by an increased mechanical sensitivity and, if the pain lasts long enough, the development of emotional comorbidities, such as depression and anxiety. However, our animals are monitored daily, and we have great experience providing food to animals that have developed hypersensitive states to ensure that they are not losing weight. We use different models of pain, from acute (the pain lasts few hours) to long-lasting, with some lasting for at least 6 months. We sometime wait for the full recovery of the pain state to collect tissue but sometime need to collect the tissue while the hypersensitive state is still present, so we can get a better understanding of the underlying mechanisms. Experiments are never continued longer than is absolutely necessary.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Our breeding protocol for genetically modified animals is mild while our pain and stress models are all moderate. However, most of our genetically modified mice will go onto one of our protocols of "moderate" severity and therefore we expect about 90% of our animals to be logged under 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 animals (male and female rats and mice) is essential for this application because we are working on a complex experience, pain, which is processed at various levels in the central nervous system and cannot be modelled in vitro. Supra-spinal systems indeed contribute to the complex physiology of pain signalling, as well as interactions with the stress hormone system. Our current approaches therefore require that whole animals are used in our experiments.

Moreover, rodents are known to display long-term emotional-like changes in long-term pain states similar to that seen in humans, such as reduced ability to experience pleasure and anxiety-like behaviour. Therefore, they are an excellent model to study the overall long-term behavioural and molecular changes that accompany the development of persistent pain states. Importantly, they are also a crucial and obligatory translational step to investigate the actions of novel treatments for persistent pain states.

Which non-animal alternatives did you consider for use in this project?

We considered the use of lower vertebrate animals such as zebrafish and of invertebrate animals such as flies and worms for this project due to their genetic tractability and faster gestation. However, literature research using Pubmed as a search engine and terms such as “pain experience”, “nociception”, “zebrafish”, “candida” indicated that these alternatives were, at least currently, not suitable to answer our scientific questions. However, this is an avenue we actively consider and are monitoring research to ensure we are using the most up to date systems with the lowest impact on harm. The use of isolated tissue was considered as an adjunct.

Why were they not suitable?

Other approaches might complement the use of animal models in this project, animal models are wholly necessary and fundamental to understanding how pain develops and its long-term trajectory. This is in large part due to the complex behaviours that can be examined in rodents versus that in lower vertebrates and invertebrates and the physiological similarities between mammals. While early studies indicate that zebrafish might be a suitable model for the study of nociception, a more peripheral mechanism of

response to a noxious peripheral stimulus, the full pain experience which involves far more complex behaviours such as fatigue, depression, anxiety and cognitive deficit must be studied in more complex organisms. If we are to begin to decipher the nature of pain and long term disease in man, this must be in an animal with similar biological circuits where human pain conditions can be reproduced with the greatest degree of similarity and therefore with significant translational value to ensure patient benefit isolated tissue is similarly not suitable in this study as we are interested in the widespread processing of stress exposure and pain across the body.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The animal number used was estimated based on the number of lab member expected to work under this licence for the next 5 years and the number of animals usually managed by one person at any one time under each main theme of the licence to ensure the success of our projects. Some experiments such as behavioural approaches often require larger n numbers that molecular approaches and under the themes of this licence, licensees will be expected to split their time equally between both work approaches. Overall, we always use appropriate statistical approaches to ensure that all our experiments are adequately powered.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

5To minimise the number of animals used, we will always ensure that all animals are monitored for the maximum number of outcome measures, within a total load that does not impact animal well-being. For example, recently, we were able to measure mechanical thresholds on a bi-weekly basis while recording emotional comorbidities on a monthly basis without any impact on animal health. At the end of each behavioural assessment which may last up to 6 months, tissue is always collected for further molecular analysis and correlation with animal behavior. We also use online tools such as the NC3R’s EDA when possible and also when requested by funders.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

First of all, our breeding is controlled very tightly with a minimum number of breeding pairs to ensure a healthy colony but no animal wastage. Animals are genotyped at a very early time point to be allocated to a treatment protocol as soon as possible. To optimize our n numbers per experiment, we use power calculations based on previous work or run pilot experiments that will then provide us with the data necessary for power calculations. We are also part of a local Animal Tissue Exchange program that ensures that all animals benefit scientific progress.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animal models

In our animal models, the severity limit will never be greater than 'moderate' and attention will be given to minimising the irritation and pain felt by the animal where possible. We will use pain models that generally cause some mechanical hypersensitivity and threshold testing procedures will be used to monitor for obvious signs of distress. When recording sleeping patterns, deviations from baseline will also be used as a welfare indicator. Experiments will not be continued for longer than is absolutely necessary. Models will be selected on the basis of their relevance to the problem being studies and attention will be paid to reducing suffering to the lowest possible level. N.B. see more information under the "Project Plan" section.

Reducing animal suffering

Our models are moderate in that they generally produce a moderate increase in mechanical sensitivity and we will not use the more invasive models unless absolutely necessary to replicate an experiment in the literature. We will use local anaesthetic whenever possible but only when this will not compromise the experiment. At the termination of the experiment animals will be humanely killed or perfused, when microscopy is going to be used to assess changes in the nervous system.

Environmental Conditions

Environmental enrichment will be provided, though this may be tailored so as not to affect experimental measurements (i.e. obstruction of the recording device), after discussion with NACWO or NVS.

Why can't you use animals that are less sentient?

The use of neonatal, juvenile and adult rodents is essential for this application because we are working on a complex experience, pain, which is processed at various levels in the central nervous system.

Supra-spinal systems indeed contribute to the complex physiology of pain signalling, as well as interactions with the stress hormone system. Moreover, rodents are known to display long-term emotional-like changes in long-term pain states similar to that seen in humans, such as anhedonia and anxiety-like behaviour. Therefore, they are an excellent model to study the overall long-term behavioural and molecular changes that accompany the development of persistent pain states.

Importantly, they are also a crucial and obligatory translational step to investigate the actions of novel treatments for persistent pain states. Less sentient animals or anaesthetised animals would therefore not provide us with a model suitable for our scientific question.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our animals are nearly monitored by ourselves on a daily basis and, on the odd occasion when they are not, BSU staff is able to reach out to us very rapidly to discuss any individual animal that may require extra care. Moreover, we have recently started using activity recording devices in the BSU cages and this allows us to monitor the welfare of our animals remotely. In the future, we will try and expand this to a maximum of our cages. We often communicate with our NACWO and the Vet and we are eager to learn about recent progress on post-surgical care. Finally, though our own research program we have recently demonstrated that using environmental enrichment and running wheels reduces mechanical hypersensitivity in injured mice and we will try and maximise the use of these tools when they do not interfere with our scientific questions.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs and RSPCA websites

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly receive communications from the 3Rs representative at our institution and also participate in 3Rs conferences. For example, one of the PhD students who will be working under this licence, presented some of our work to the latest 3Rs conference in London in April 2023. We also communicate on a daily basis with BSU staff and listen to their suggestion for any novel 3Rs implementations.

83. Neurobiological mechanisms of risk in psychiatric illness.

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

psychiatric illness, genetics, behaviour, brain, neurophysiology

Animal types	Life stages
Rats	adult, juvenile, neonate, embryo, pregnant
Mice	neonate, juvenile, adult, 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?

The project aims to improve our understanding of the biological mechanisms by which genetic and environmental factors increase the risk of psychiatric illness. The research aims to contribute new data leading to the development of better diagnosis and treatment based on causality.

We will use animal models as a part of an integrative research programme with the following objectives:

1. To identify the changes in brain functions linked with known genetic and environmental risk factors.

2. To identify new treatments for mental illness based on the causal links between risk factors and brain dysfunction.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Mental health conditions are one of the main causes of the overall disease burden worldwide (over 20% of years lived with disability) and a growing concern for public health. According to WHO, an estimated 1 in 8 people live with a mental disorder worldwide, and, by the age of 75, approximately half of the world population can expect to develop a mental disorder. In particular, psychiatric disorders including schizophrenia and autism are three of the top ten causes of disability as rated by the WHO. In England alone, the socioeconomic cost of mental health conditions, taking into account both the health and social care costs and lost earnings through decreased employment is projected to increase to £88 billion by 2026.

However, due to an insufficient understanding of the causes and neurobiological mechanisms of mental disorders, the progress in the diagnosis and treatment of these conditions has been disappointingly small compared to other areas of medicine. In particular, there are no effective therapies for autism, whereas the treatment for schizophrenia has not advanced substantially since the introduction of neuroleptic medication which also has significant side effects. Furthermore, the current psychiatric diagnosis framework is largely descriptive and based on heterogeneous and overlapping disease categories, due to insufficient diagnostic markers. Recent human genetic and epidemiological studies in psychiatric disorders have contributed significantly to the understanding of the multiple possible causes of these conditions. On their own, these findings do not improve the treatment of these conditions. To obtain a better diagnosis and treatment of mental illness, we need to know how these risk factors change the brain's functions. The project licence described here aims to meet this need.

What outputs do you think you will see at the end of this project?

The research will bring new knowledge about the functional impact of psychiatric genetic and environmental risk factors on brain functions at the level of individual brain cells, cellular ensembles and circuits. The findings will help understand how these functional alterations relate to mental illness symptoms and will contribute to a rational basis for the development of better diagnostic markers and therapies. The results will be published in scientific journals and communicated at learned societies, aiming to reach a broad audience in the fields of neuroscience, neuropsychiatry, and pharmaceutics.

We anticipate that this work will lead to the discovery of new drug targets and the development of new treatments in these conditions, with the potential for new collaborations including with the pharmaceutical industry.

Who or what will benefit from these outputs, and how?

An essential stage in the development of new treatments and objective diagnostic markers is the generation of appropriate models of high construct validity, which is the objective of this application. The benefits of this process will be: 1) to advance our understanding of the biological basis of major psychiatric disorders, and 2) to provide validated models suitable for screening novel pharmacological interventions.

The project will lead to the further characterisation of novel rodent models of confirmed risk-associated genetic modifications in key genes implicated in the risk for psychiatric disorders.

The outputs include novel animal models of genetic risk factors for psychiatric conditions. We will characterize the behavioural and functional consequences of these genetic alterations at molecular, single brain cell, brain circuit, and systems level. The models will be made available to other academic groups and the results of our studies will be published in Open access format in peer-reviewed journals.

These outputs will benefit the research community in the fields of neuroscience and neuropsychiatry, by expanding our knowledge and understanding of the mechanisms of mental conditions. In the short- and medium-term, the data will provide support for funding further research projects. In the long-term (e.g., upon completion of the project) the results will help springboard future collaborations including with the pharmaceutical industry and neuropsychiatric clinic.

How will you look to maximise the outputs of this work?

To maximize the outputs we will disseminate findings through communications at learned societies, and publications in peer-reviewed scientific journals aimed at broad audiences in the fields of neuroscience, neuropsychiatry, and more generally in medicine. Results of wider public interest will be communicated through public engagement channels.

When unsuccessful approaches or negative results are likely to have a strong impact on the current knowledge and experimental approaches in the field, they will be included in the publications alongside the other relevant data. This will inform other researchers in order to avoid repeating strategies that are unlikely to generate progress in the field.

Species and numbers of animals expected to be used

- Mice: 4000
- Rats: 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.

The chosen rodent species uniquely combine genetic flexibility and an established set of behavioural tests. Furthermore, rodents are suitable for functional and molecular investigations. Rats are used in addition to mice because of the extensive range of available experimental behavioural tests.

The central aim of this project is to understand the neurobiological mechanisms underlying the genetic and environmental risk for psychiatric illness. The principal measures used in the project are derived from behavioural testing and therefore most of the studies will be used in animals in adult stages of life. Interactions between these genetic and environmental risk factors (e.g. immune/inflammatory stressors) during late pre-natal

stages and early post-natal life can shape the age of onset and the natural evolution of the disease. To address this neurodevelopmental dimension of risk we will also perform studies involving early life manipulations on pre-natal stages or on post-natal, juvenile animals. The post-natal manipulations may involve limited periods of separation of pups from their mothers, or moderate stressors during adolescence (post-weaning).

We will apply the most refined methodology available in all studies. In behavioural testing we will use food or water restriction only where necessary to motivate behaviour, and for the minimum duration needed to complete the study. Functional studies will use techniques that minimise welfare costs, e.g., ex vivo electrophysiology recordings. When invasive brain surgery is needed, we will use anaesthesia, pre-operative and post-operative analgesia (including topical analgesia) as needed, and aseptic surgical methods to avoid post-operative complications. When required, animals will undergo surgery for a single administration of genetic viral vectors or chronic implantation of cannulae and will be freely moving upon return to home cage.

We will maintain close links with our animal support and veterinary staff across all our studies.

Typically, what will be done to an animal used in your project?

We will breed genetically altered rats and mice. These animals will allow us to measure the effects of genetic risk factors for mental illness on clinically relevant animal behaviour and brain function.

These genetic changes are typically not harmful in rodents. However, some can increase animal vulnerability to stress. We will adapt the handling and maintenance of these animal lines to avoid stress.

In some studies, we will investigate the effects of environmental risk factors in early life such as the effects of maternal immune activation on offspring, maternal separation during postnatal life, or early life stress induced with a choice of stressors: elevated platform exposure, restraint or mild electric footshocks. In these studies, induction of a moderate level of stress is important for us to understand how these genetic risk factors might affect an individual’s vulnerability – or resilience – to environmental factors.

We will evaluate the behaviour of these animals using typically mildly aversive tests. These include measurements of simple reflexes, basic motor skills, performance in maze and in lever-pressing tasks. We will also measure levels of anxiety and emotional reactions. In some animals, we will test their ability to learn the association between environmental aversive stimuli (e.g., between sound and a mild electric footshock) using Pavlovian fear conditioning. This paradigm provides objective measures of behaviour necessary to understanding how genetic and environmental risk factors contribute to the development of learning and memory deficits in psychiatric conditions (leading, for example, to aberrant attribution of importance to irrelevant events, associated with psychoses).

Some tests may require restricted food or water intake to motivate behaviour. To ensure the health of the animals, we will monitor and keep these restrictions within safety limits.

Investigation of the causes of altered brain function and finding potential new remedies may require direct manipulations and measurements of brain cell activity in relevant brain

regions in a limited number of animals. We will implant devices to administer compounds, genetic vectors (viruses) to express marker proteins, or measure cell activity. The procedures may produce some discomfort or pain. We will use general anaesthesia, aseptic surgery, pre-operative and post-operative local (and topical) analgesia to minimise animal suffering. We will closely monitor any signs of distress. After recovery from surgery, the animals will complete behavioural testing to quantify the effects of the treatment. In some of these animals, we will record the activity of brain cells during the experiment.

At the end of testing, all animals used in the licence will be killed by a humane method under sedation. Often, we will collect brain tissue for molecular or functional analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals may experience mild acute pain, or weight loss, mild dehydration, hypoglycemia and fever lasting for up to 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 expected severities are mild (60%) and moderate (40%) for both rats and mice.

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are two main reasons why animal use is necessary to understand the mechanisms through which specific genetic risk factors confer enhanced risk for neuropsychiatric disorders.

The first reason is because these conditions fundamentally involve disordered activity in brain circuits. No currently available in vitro model systems can capture the full range of molecular, cellular, physiological and behavioural consequences of genetic and environmental risk factors for neuropsychiatric disorders. It is therefore necessary to use animal model to understand both normal and abnormal function in these brain systems. The second reason is that it is not possible to directly access the diseased tissue (i.e., the brain) in human disease/mutation carriers during life. Non-invasive imaging techniques in humans can provide some information about altered brain structure and function, and such approaches form a part of our current research programme. However, these techniques

are fundamentally limited in their scope and cannot be used to study the basic physiological and molecular properties of diseased circuits.

Therefore, animals are uniquely suitable to interrogate the consequences of genetic and environmental risk factors on functional alterations at single brain cell, brain circuit and system levels, and correlate these findings with behavioural and cognitive changes of clinical relevance in the same organism. An essential stage in the development of new treatments is the generation and characterisation of appropriate animal models that more closely reflect the biological alterations associated with neuropsychiatric conditions, which is the objective of this application.

Which non-animal alternatives did you consider for use in this project?

We have considered two emerging non-animal alternatives for studying the biological mechanisms of psychiatric illness: in vitro cell and organoid culture models, and in silico approaches.

In vitro cellular and organoid models allow the rapid screening new disease-modifying drugs using functional investigations at the cellular level. By comparison to genetic animal models, genetic interventions are easier to implement in cultured cells and organoids with shorter delays until their

functional effects at cellular and molecular levels can be measured. In addition, human patient-derived in vitro cell cultures bear the genetic make-up of the patient and therefore are a significant step towards personalized medicine.

In silico approaches generate mathematical models of biological processes based on experimentally acquired data, to predict the consequences of expected changes in well-defined model parameters. These approaches have found broad applicability in modelling molecular interactions, behaviour and disease.

We recognise the importance of in vitro and in silico models as complementary tools in our research arsenal, given their suitability for rapid drug screening and predictive capacities. Our parallel in vitro research programme takes cells derived from both animal models and human mutation carriers (eg iPS cells) to investigate cellular development, differentiation and biochemistry/physiology. We have adopted a holistic, integrated and translational approach in our programme, where we conduct research in animal models in parallel with studies in human cell models, computer simulations, and non-invasive imaging and behavioural approaches in human patients. These different levels of analysis will inform one another, limiting wastage. This integrative approach will allow us to apply replacement methods when they are appropriate, and will facilitate the translation of our findings to clinic.

However, the animal component of our study is necessary to fully understand mechanisms at the circuit level, and to bind together biochemical, molecular, physiological and behavioural investigations. Furthermore, the validation of our animal models will provide an essential step in developing valid animal model systems for the future development and testing of novel interventions for these disabling disorders.

Why were they not suitable?

In vitro cellular models (e.g. 2D culture monolayers, 3D organoids and organ-on-chip technologies) do not fully replicate the natural development of brain tissue, and do not fully reproduce vastly more complex circuit systems of the brain as a whole. These models are also limited by the inclusion of contaminating components of animal origin (e.g. culture medium sera and scaffolds), although development for animal-free systems is ongoing. In the field of neuroscience, 3D organoid, and organ- on-chip technologies have only recently seen some advances in replicating a limited set of brain structures as the blood-brain barrier, retina, and neuromuscular systems for the study of amyotrophic lateral sclerosis.

Critically, the findings at single brain cell level obtained in cell and organoid culture models do not immediately translate to disease-associated functional alterations at systems levels including, for example, behavioural and cognitive deficits. This indicates that in vitro models are better regarded as an important bridge between traditional cell cultures and animal models. The ability to integrate information across several scales, from cellular to systems and behaviour, in the same organism is uniquely provided by animal models.

In silico models rely on assumptions about biological variables that need to be experimentally verified or controlled, and therefore require validation obtained through direct experiments using in vitro or 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 estimated the number of animals that will be used based on prior pilot studies performed in our laboratory during the current licence period, and on results from our laboratory and world-wide, published 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?

We will use appropriate statistical and experimental design tools such as the NC3Rs Experimental Design Assistant (https://eda.nc3rs.org.uk/) to optimise the number of animals used in each study.

Experimental design will be based on the extensive experience of the investigators involved in the programme in the techniques described. Power calculations will be performed for all experiments using appropriate power calculation instruments (e.g. the Lenth Power and Sample Size calculation software (http://www.stat.uiowa.edu/~rlenth/Power; Lenth, R. V. (2006-9)). Power calculations will ensure that the minimum required number of animals to obtain a statistically robust outcome is used in each experiment.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will take a number of additional steps to minimise the number of animals used in this project:

1. We will only generate novel transgenic lines where no existing suitable lines are available and will screen relevant databases for this purpose.

2. Breeding will be carefully matched to experimental requirements.

3. Breeding will be optimised wherever possible to produce only the genotype required.

4. Cryopreservation of gametes and embryos to archive lines will be used where appropriate to avoid wastage from the need to maintain colonies by continuous breeding.

5. Where appropriate, non-invasive techniques delivering multiple relevant outputs (eg MRI scanning to look at structure and chemistry) will be utilised.

6. Where appropriate without adding to severity, additional data outputs will be collected from experimental animals (e.g., post-mortem, tissue for gene expression analysis, ex vivo tissue for functional analysis).

7. We will include data from the relevant genotypes and wild-type littermates of both sexes.

8. The extensive experience of the investigators applying the techniques in this licence will help minimise the 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.

We have chosen mice and rat models to investigate the behavioural and physiological effects of genetic mutations associated with neuropsychiatric disorders. Mice are widely used for work involving genetic alterations. The standard protocols, methods and reagents have been optimised for this species and there are acknowledged benefits from their use. There is considerable local expertise in relevant mouse behavioural models and physiological measures. Mice currently offer the greatest range of flexibility in terms of genetic manipulation. However the development of transgenic rat models is rapidly advancing (e.g. using CRISPR-Cas9 technology). Rats have advantages in terms of their better characterised behavioural repertoire.

Suffering will be minimised by focussing our characterisation of these models on established behavioural measures and ex vivo characterisation e.g. of molecular and

physiological changes. These measures will provide an essential baseline against which future investigations can be planned.

Why can’t you use animals that are less sentient?

Behavioural testing requires animals at adult stage.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will follow the NC3Rs guidelines for anaesthesia and stereotactic surgery and post-operative care [https://www.nc3rs.org.uk/3rs-resources/anaesthesia; https://www.nc3rs.org.uk/3rs-resources/refining- rodent-stereotactic-surgeries].

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the NC3Rs guidelines for anaesthesia and stereotactic surgery [https://www.nc3rs.org.uk/3rs-resources/anaesthesia; https://www.nc3rs.org.uk/3rs-resources/refining- rodent-stereotactic-surgeries].

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will visit the NC3Rs web site regularly as the principal source of information regarding advances in the 3Rs, which includes peer-reviewed publications in this domain. We will refine our procedures by implementing NC3Rs recommendations as they become available. For housing and handling refinements we will maintain close communication with our animal support and veterinary staff.

84. Translational Cancer Molecular and Cellular Biology

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Cancer, Molecular biology, Novel therapies, Cell biology, Tumourigenesis

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 better understand the molecular and cellular processes within mammals, that when deregulated, result in cancer/tumour formation and furthermore, allow tumours to grow and spread.

This novel insight and knowledge gained through this project will help generate new therapies that we can take to (ie translate to) the clinic for significantly improved patient benefit.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 we have made significant progress in treating some types of cancer successfully (e.g. certain childhood leukaemias) most other cancers remain deadly and collectively represent one of the top causes of human mortality each year. Over the last two decades, investigations into the basic cellular and molecular biology of cancer have given us ground-breaking insights into this specific disease biology.

We have discovered that cancers that develop in a particular organ (e.g. lung, breast), represent multiple different types of cancers, each of which develops differently and needs distinct therapeutic strategies/interventions. Added to this we have also established that even within a single tumour in a single person, there are multiple different genetic changes that have occurred while that tumour developed and we are currently unable to say whether a particular therapy will be equally effective for each of these genetic variants. Thus, cancer remains an incredibly complex and context-dependent disease that requires detailed scientific research to identify new strategies that will eventually prevent cancer from being a life-threatening disease.

In vivo research and experimental models of disease, are key to obtaining the correct critical context- dependent knowledge on the development of cancer and its metastasis. Thus enabling the development of patient-specific tailored therapies. Therefore, in vivo research is one of the most important tools for biomedical research and essential for the development of new medicines and therapeutic strategies to save the lives of cancer patients.

What outputs do you think you will see at the end of this project?

At the end of this project, we will obtain a significant level of novel insight into how cancers develop and disseminate (metastasise) throughout the body and also how we can detect these cancers early and challenge them with newly developed therapies.

We believe that such impactful findings and progress in our understanding of cancer biology in vivo will be of great interest to the wider public and scientific community and thus enable initiation of a Phase 1/II clinical trial and high-impact publications in top tier journals with large international readerships.

Who or what will benefit from these outputs, and how?

Overall, it is hoped in the long term, this research will potentially enable the identification and selection of specific gene targets in cancer patients who will benefit from the specific targeted therapies and combination immunotherapies this project aims to develop. Briefly,

1. We will utilise the mouse models/in vivo studies to identify new biology/genetics (such as new tumours suppressor genes and oncogenes) that are involved in cancer initiation, development and metastasis. These same mouse models and in vivo studies will then also be employed to identify and develop new cancer therapeutics and treatment modalities.

2. With respect to the development of new improved specific targeted therapies and combination immunotherapies, it is important to make sure these will be highly specific/targeted therapies. Thus, these mouse models and studies will be invaluable as we will be able to utilise them to assay that our new drug/compound and therapies target only the tumour cells/tissue and not harm other normal tissue and organs in the mouse and importantly minimise pain suffering or lasting harm to the animal.

3. The long-term benefits of steps 1 and 2 above will be for cancer patients and cancer biomedical research and the health professionals' care systems. Our new genes and biological targets we identify and characterise will be used as both biomarkers and targets for the new therapies we develop and also validate in this project of work. The new basic cancer biology understanding we will obtain from this project of work will be published and presented at international meetings and other media sources; so as to enable the greater research community to use the data to enhance related world-wide research into cancer biology and therapeutics and thus ultimately patients benefit world-wide.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this work through collaborations within and beyond the Institute/University, presentation in scientific conferences and by sharing the data with our collaborators. Also, publications of results (negative or positive) in high-impact publications in top tier journals.

Species and numbers of animals expected to be used

- Mice: 6,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are interested in understanding better the processes that lead to human cancers initiating, developing and spreading, in order that we can develop therapeutic strategies to treat cancer and that we can translate to the clinic. The mouse has many virtues that mean it is an ideal model for our studies. Thus mice have:

-been used successfully for decades by scientists to develop effective drugs in the clinic.

-been used successfully for decades by scientists to understand some of the key biology of cancer development that we know today.

-a breeding-cycle that is relatively quick so important advances in the understanding of cancer biology can be made as soon as possible.

-many genetically altered (GA) versions available to make scientific research efficient, relatively quick, and require fewer animals. The technologies to generate further GA mice that possess more relevant gene modifications is now routine and provided through commercial sources which limits the errors by less experienced staff in making these animals. for example we will use mice with either normal or defective immune system that are supplied by authorised breeders. Animals with defective immune system will be used for growth of human cancer tissue or cancer cell lines to determine both efficacy and mechanisms of action of our novel therapies

With the exception of mouse strain genotyping, which needs to be done early (usually on neonates, juveniles if repeats required) we are only interested in processes that occur mostly in adult humans. Thus we use adult mice for our studies.

Typically, what will be done to an animal used in your project?

All experiments require a pre-planned series of procedures that are designed to maximise the data from the use of mice while using the minimum number of mice and imposing the minimum amount of discomfort and harm.

Typically, cancer cells will be injected into a mouse to allow a tumour to form. Alternatively, some mice have been genetically engineered to develop tumours through induction, which faithfully recapitulates the human disease.

Imaging may be incorporated to any study carried out in this protocol and will generally be used to confirm that most animals are developing detectable tumours before the start of treatment. Imaging may also be used to follow up the response of the tumours to treatment. This would also reduce the number of mice we use, as it will allow us to study, in the same experiment, short-term and long-term effects of therapy. Imaging is carried out under anaesthetic, using modalities such as Magnetic Resonance Imaging (MRI) or Bioluminescent imaging (BLI), both standard imaging techniques used widely in animal-based research. Blood samples may be collected to monitor the animal's health or the effect of the treatment. A volume of 50 microlitres (a drop of blood - one microlitre is one-millionth of a litre) is normally sufficient for this purpose. Generally, we would expect to run these checks up to once weekly over the course of an experiment and also at the end of a study where the mouse could have more blood removed under terminal anaesthesia from which they would not be woken. The blood sampling will not exceed what is indicated in the guidance from NC3Rs https://www.nc3rs.org.uk/blood-sampling-general-principles.

In some instances (dependent on the model used, ranging from weeks to months for some slower- growing genetic models) the primary tumour may then be removed surgically to allow metastases (sites of secondary tumours) to form, typically in the lung, liver and bones. This would be performed under anaesthetic and with pain relief given, according to the advice given by our Named Veterinary Surgeon (NVS).

Mice will be given different treatments, either as single agents or a combination of agents to try and find the best regimen that could be applied in the clinic. These could be administered by topical application, direct injection into the tumour or through delivery via oral or subcutaneous intratracheal administration, or the bloodstream or into the peritoneum, or dosed feed in diet or drinking water.

Some studies require surgical procedures such as the injection of cancer cells directly into an organ (for example, the lung). For these studies, animals will be anaesthetised prior to and during surgery and treated with pain medication to relieve the feeling of pain post-surgery. Aseptic techniques are routinely used and we use approved anaesthetics and pain medication after discussions with our NVS.

Mice bearing any of our cancer models will be culled at a pre-determined time-point whenever possible. Implanted tumours tend to develop and grow at a more predictable time frame than genetically altered (GA) mice developing induced tumours. Thus GA mice experiments often require several weeks to months (depending on the study and model) before tumours arise and experiments can be conducted, whereas implanted tumour

studies are often completed in 3 months. Mice are monitored routinely throughout taking into account various factors such as appearance, body weight and behaviour.

What are the expected impacts and/or adverse effects for the animals during your project?

From our past experience, we predict that the planned studies will cause mild or moderate discomfort. Animals are expected to return to normal activity within minutes after injections. When animals have been subjected to surgery to administer cells, the recovery time is normally longer (2-3h) however mice usually show little change in their general behaviour. To support animals recovering from surgery we provide a softer easier to digest diet placed within easier reach for at least the first 24hrs following the procedure.

During the advanced stage of tumour growth, some distress may occur, potentially affecting normal bodily functions.

Daily observations will be conducted to assess the animals' normal behaviors and the overall condition of their cages and environment. If an animal exhibits signs of distress or suffering, such as subdued behavior when provoked, weight loss exceeding 15%, persistent weight loss over time without improvement, or continuous hunched posture or piloerection, it will be humanely euthanized.

At the conclusion of the study, after collecting all necessary data, all animals will be euthanized using humane procedures, and their tissues will be utilized for analysis.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

In our breeding protocol we do not expect any clinical effects and from experience this protocol is of mild severity with the majority of animals experiencing sub-threshold severity. A majority (90%) of the animals used in our experimental protocols 2 and 3 will be tumour-bearing and most will be subjected to single agent or combination therapies with the aim of eliminating their cancers or preventing return of the cancer. We expect 25% of the animals will, in addition, undergo some form of imaging to assess their tumour burden. For these reasons, a majority of animals under these protocols are expected to experience moderate severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice are used as excellent subjects with respect to recapitulating human disease as faithfully as possible They are genetically/biologically similar to humans and thus susceptible to many of the same health problems including cancer. Also, they have short life cycles so they can easily be studied throughout their whole life span or across several generations.

With respect to the overall aims of this project, the use of GA mice with precise targeted genetic defects (such as Limd1 gene knockout or KRas activation) will allow us to accurately focus on specific aspects of cancer biology and its development as summarised in the Aims section above. Simply put, using such a GA mouse model we can specifically target various genes and aspects of the mouse biology that we know are altered in human cancers and determine how these contribute to tumour formation and metastasis and also use these same models to help identify potential new anti-cancer therapeutics.

The use of GA mice is also required for the analyses of the complex tumour immune cell microenvironment. Currently, this can only be examined using in vivo models such as GA mice where we have genetically manipulated key tumour suppressor genes and oncogenes in the context of GA mice that are both proficient and deficient for an active immune system and anti-tumour response.

Which non-animal alternatives did you consider for use in this project?

For the purposes of our project, we primarily rely on non-animal alternatives. The majority of the work we do in the lab involves the in vitro culture of human cell lines, and we employ a variety of methods, including 2D assays (adhesion, migration), biochemical analysis (e.g., western blotting), immunological techniques (e.g., flow cytometry), and molecular research (transcriptomic, genomic, and proteomic).

These analyses use cells that have been genetically, immunologically, or pharmaceutically modified to focus on particular goals. As a result, only a small portion of the research conducted at our centre or institute uses animals. Additionally, we make extensive use of sophisticated multi-cellular 3D models created in vitro that are intended to answer fundamental questions and replace animal models.

Why were they not suitable?

The non-animal (in vitro alternative) are indeed suitable for specific aspects and questions of tumour biology and generate valuable knowledge. However they fail to model critical aspects of the biology of novel anti-cancer agents and the complexity of a live tissue served by blood vessels and lymphatics and having innate and adaptive immune cells. These aspects can only be assessed in realistic models of cancer in whole animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on our extensive experience and data collected over the past 10 years, we will use a total of 6,000 mice in this project. This includes 3,000 for mild breeding of genetically modified animals, 2,000 with inducible tumors, and 1,000 with implanted tumors. These quantities have been carefully selected to ensure reliable data generation in murine cancer models.

The objective of this project is to advance our models and therapies by studying the interactions between novel treatments, the tumor microenvironment, and the host immune system. These investigations will involve the 6,000 mice and enable us to explore the fundamental biology of cancer initiation and novel therapeutics. Each study will determine the precise sample size required to achieve statistical significance.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

10+ years of experience in designing, running and publishing in vivo studies means I am well prepared for training and supervising my staff in effective means of designing and executing in vivo studies.

First we start with a clear hypothesis. This is essential so that we can design an experiment with defined goals, sufficient numbers of mice, a clear monitoring strategy, a pre-planned means of analysis, a randomisation plan and a data blinding plan. All of this should be defined and agreed before the experiment commences.

For those experiments where a therapeutic change is desired, we would first conduct multiple in vitro studies including, if possible, 3D multicellular pathology-mimicking models in order to prove that indeed, there is a potentially useful biological effect. Only then would in vivo experiments be considered. Sometimes the justification may come from studies from other laboratories. The level of effect helps to determine the expected % biological change that will inform the power calculations.

For many years we have used successfully the on-line power calculation tool (http://biomath.info/power/). Our resulting experiments have produced data that provide statistical significance and thus more scientific value.

In preparation for this license I have become familiar with the excellent NC3Rs EDA tools. I will be introducing these tools to all of my team with the instruction they should become familiar and where valuable, to use them in their experimental designs. The NC3R EDA tools can improve experimental design and increase my staff's knowledge and understanding of in vivo studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

By first exploring conditions in small pilot experiments the final conditions can be optimised and also reduce the number of animals subjected to unexpected side-effects. This is done in consultation with our in-house statistician team, who has done the power calculations to estimate the number of mice that we will require.

Wherever possible use of in vitro assays will reduce the number of animals required and we routinely use data from our past studies to estimate the minimum number of animals

required to address the stated primary endpoint of the experiment without having to repeat entire studies

The studies will only include agents that have been pre-selected as potential new therapies using our sophisticated in vitro and ex vivo models of cancer as detailed above.

We have incorporated in vivo imaging in our experimental protocols to enable smaller cohorts of animals to be observed prospectively throughout an experiment rather than relying on killing of separate groups of animals at each time point. This is especially informative for lung cancer models, where estimation of tumour burden can be very informative using imaging. The use of imaging in our protocols will avoid the waste of entire cohorts of animals.

When possible, the same groups of control animals (untreated or mock-treated) will be used in several studies to minimise the number of animals. Tissue is and will be harvested from each study for laboratory investigation to collect the maximum available information from each animal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use the following types of animal models: -

Immunocompromised mice will be typically used as they will allow us to assess the growth of human cancer cells in an animal model, as well as enable us to use genetically modified cell lines we have created in the lab as part of the in vitro analysis (syngeneic studies) of mechanisms of tumour growth, thus reducing experimental variability.

Depending on the study needs there are several different immunocompromised mice we can use (e.g. Athymic nude mice, BALB/c nude and NOD SCID). It is envisaged that most studies using immunodeficient mice will predominantly use these strains but this project will not be limited to just these and others may be utilised that have different aspects of their immunity modified or ablated. The utility of a repertoire of Immunocompromised mice in tumourigenesis assays means that we will be able to use these to help pinpoint different aspects of the immune system that are involved in tumour development and spread. Thus, gaining significant insight in the important file of immune-oncology and related therapeutics.

Genetically engineered mouse models (GEMM) with a C57/BL6 background will also be used in the project as they have a partly intact immune system which allows the assessment of the effect of immunotherapy on the xenografted cells.

In addition, we will also use GEMM in tumour development. Such mice and studies will enable us to directly correlate our findings to the development of novel biomarkers and targeted therapies for human cancers.

The methods we will use include growth of cancer cells under the skin of the animal which is minimally invasive and cause limited suffering and distress. When tumours form they are easily visible and can be injected directly with the therapeutics causing only mild to moderate distress. However, we will also use models that better represent human tumour growth. For example, the most simple of these involves the injection of tumour cells intravenously to generate lung metastasis providing an accurate model of human lung cancer. We have also developed orthotopic tumour models, where cells are injected directly into the relevant organ, e.g. the lung or breast fat pads. The tumours that develop accurately recreate the complex tumour-stromal interactions seen in human disease. Although these models require more invasive procedures, the animals recover very rapidly (within hours) and the administration of analgesic reduces pain and limits distress. Furthermore, results from orthotopic tumour models or genetically altered animals with induced tumour formation will generate more relevant information that in the long-term will reduce both the number of studies and the suffering of the animals.

All our studies are classified as mild to moderate.

Why can’t you use animals that are less sentient?

We prioritize the use of adult mice over zebrafish, Drosophila (fruit flies), or C. elegans (nematode worms) for our cancer biology research due to their greater genetic and physiological similarities to humans. Adult mice possess organ systems and functions that closely resemble those of humans, allowing us to replicate and investigate cancer-related processes accurately.

While other model organisms have their advantages, adult mice provide a more appropriate and informative model system for our specific focus on cancer biology and the need to replicate human-like processes. Using adult mice allows us to obtain accurate and comprehensive insights into the biology of cancer, as their genetic makeup, physiology, and anatomical features closely resemble those of humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have put in place specific measures and ongoing improvements to improve the care and welfare of the animals used in these studies. These consist of:

Aseptic procedures and analgesia are used during surgeries and implant procedures to minimise the risk of infection and provide pain relief.

Increased monitoring and post-operative care: We closely monitor the health of the animals, offer thorough post-operative care, and effectively manage pain.

Early tumour detection: Non-invasive imaging makes it possible to identify tumours at an early stage, allowing for early intervention and lessening animal suffering.

We promote the use of ultrasound echo-guided injections to deliver medications directly to target organs or tissues, reducing the need for surgical intervention and potential animal distress. This method aids in preventing confounding influences on experimental findings.

For animals with compromised immune systems, we also offer additional and specialised care. These animals' compromised immune systems necessitate additional care to maintain their well-being. To protect their health, strict hygiene rules, careful monitoring, and infection control procedures are in place.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There is a significant body of available information and literature on the guidance for best practice which we will follow to ensure we conduct our studies in the most refined way.

Here are important references (example and not limited to these) that we always consider with respect to best practice guidance : -

1. 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

2. Refining procedures for the administration of substances, Report of the BVAAWF/ FRAME/RSPCA/UFAW Joint Working Group on Refinement, D.B.Morton et al; 2001.

3. A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes, Diehl et al, J.Appl. Toxicol. (2001) 21, 15–23.

4. The directions for reporting of actual severity levels, for example; Smith et al., Laboratory Animals 2018.

5. Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0 Percie du Sert N, Ahluwalia A, Alam S, Avey MT, Baker M, et al. (2020) Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0. PLOS Biology 18(7): e3000411. https://doi.org/10.1371/journal.pbio.3000411

6. M.F. Festing, Design and statistical methods in studies using animal models of development, ILAR J, 47 (2006) 5-14. 18

7. J. Charan, N.D. Kantharia, How to calculate sample size in animal studies?, J Pharmacol Pharmacother, 4 (2013) 303-306

8. LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. A report by the LASA Education, Training and Ethics section. (E Lilley and M. Berdoy eds.) http://www.lasa.co.uk/publications/

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As the project licence holder, I will have regular discussions with the Named Persons and animal technicians in our institute to review current approaches and whether there are any new 3Rs opportunities. I will subscribe to the NC3Rs e-newsletter. These monthly updates

focus on funding opportunities, 3Rs events and publications and thus will be very useful in keeping me informed of such advances.

I will also attend NC3Rs events and workshops so as to keep abreast of 3Rs advances/approaches and register for NC3Rs upcoming webinars and watch the recordings of past webinars, including our series highlighting researchers from across Europe that take place each year.

85. In vivo compressive forces

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cell migration, cancer, nucleus, mitosis, mechanics

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?

To use Zebrafish as a model, relevant to human disease, to study in vivo mechanical stress.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

When they divide or migrate, cells in tissues have to push against other cells around them or squeeze through very tight spaces.

Studies in cell culture where cells were forced into very small spaces, such as very small channels made of silicone (microchannels), showed that single cells that are physically compressed experience mechanical stress. This can lead to DNA damage because of deformation and rupture of the cell nucleus or to errors during cell division. As a consequence, mechanical compression can also cause changes in the DNA of cancer cells which can be inherited and may contribute to cancer.

However, whether in vivo (in an intact animal or tissue) compressive forces can affect cell divisions, cell differentiation or cause heritable DNA damage has not been yet investigated. Indeed, it is challenging to image migrating cells in confined environments in mammalian tissues as these need to be surgically exposed. Moreover, it is difficult to manipulate the physical properties of tissues in vivo.

My research team and I will overcome these challenges by using the Zebrafish. As its embryo is small and transparent, we will be able to visualise migrating cells directly in an intact animal that is suitable to mechanical manipulations. We will investigate a population of embryonic multipotent stem cells (cells of the embryo that can differentiate into many different tissues) called neural crest cells. These cells are essential to development of all vertebrates, and differentiate into many kinds of tissues, including cartilage and bone, pigment cells of the skin, neurons and glia. In the trunk of the Zebrafish embryo, we observed that neural crest cells migrate through a narrow space in between other tissues before differentiating into glial cells and neurons. This confined migration is highly conserved across vertebrates, including humans. We have observed that neural crest cells experience significant nuclear deformation during migration. Moreover, they divide asymmetrically, giving rise to daughters with different fates, and, importantly, sometimes they make errors when dividing.

These findings are important for human health, because trunk neural crest can initiate neuroblastoma, a common solid tumour in children. Neuroblastoma still has only 50% survival rate and in 98% of cases is linked to chromosomal abnormalities rather than germline mutations. It is unknown why neuroblastomas suffer genomic errors. One possibility is that this might be a consequence of in vivo mechanical stress.

Using the Zebrafish as a model we will dissect, using a multi-scale approach, whether physiological mechanical compression can cause DNA damage in cells within a developing vertebrate embryo. Our work will elucidate whether physical stress can contribute to human cancer initiation, and will shed light on the origin of chromosome damage in cancer.

What outputs do you think you will see at the end of this project?

The proposed research aims at understanding whether mechanical stress occurring during in vivo embryonic development contributes to DNA damage and mitotic errors, and whether such mechanical inputs could contribute to cancer initiation in vivo.

This project will be transformative for a field that has so far heavily relied on in vitro microfabrication approaches to understand the consequences of mechanical stresses on cell function (i.e. nuclear integrity, mitosis, cytoskeletal organisation, cell fate specification), and the publication of findings in a physiological in vivo model such as Zebrafish neural crest cell migration will finally address this gap.

Who or what will benefit from these outputs, and how?

The immediate beneficiaries of the proposed research will be other researchers working in the UK and internationally in the fields of tissue mechanics, cell migration, developmental biology, cancer cell biology. My research will pioneer novel strategies to manipulate tissue forces in vivo in Zebrafish that will be useful to other researchers working in the field of developmental mechanics. My collaborators will directly benefit from the research 1) by co-

authoring the scientific publications that will result from it; 2) by exploiting any relevant findings for further investigations in the future.

In addition, the implications of our research will be relevant to clinical and non-clinical researchers studying cancer initiation , metastasis, potential therapeutic targets and drug resistance mechanisms, as well as other key stakeholders: (a) clinicians, healthcare professionals and public policy makers; (b) pharmaceutical companies looking to translate findings; (c) students and researchers receiving lab experience and training in skills in in vivo research; and (d) the lay public through outreach initiatives.

How will you look to maximise the outputs of this work?

Before publication, the results of the research will be discussed with the research community at specialised national and international in-person and online conferences as well at local seminar series.

The results of our research will be published in the appropriate specialized journals. Upon submission to journals, articles will be made available to the scientific community as preprints by uploading them to preprint servers . Upon publication, papers will be advertised to their readership via official press releases, via community websites and on social media.

Moreover, my laboratory will also generate novel genetic tools and materials that will be made publicly available upon publication (or earlier, upon reasonable request) and will shared with other researchers via community resources.

My research team and I also believe that engaging with the non-academic public is an essential part of the work of a researcher. While publishing work in specialised journal benefits the science community, it is important that the wider public understands and trusts the work we undertake as scientists. We intend to keep engaging with the local community to ensure the implications of our research are well understood by the wider public.

For example, my lab will participate at science festivals. We plan to develop activities that help non- academic audiences understand what the consequences of mechanical stress on cells are, and why is it important that we understand this to fight cancer.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 12,008

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 zebrafish embryos for this research because they are transparent, develop rapidly and we can carry out genetic and mechanical manipulations in these small embryos. The neural crest cells are formed over approximately 24 hours. This means that we can put the intact embryo under a specialised microscope and can easily image the whole process of vertebrate neural crest migration within the course of one experiment.

Neural crest cells are found in all vertebrates and the mechanisms of neural crest migration are conserved in humans. All of our experiments will be carried out on embryos under 5 days old, which do not require a licence from the Home Office. To generate these embryos, it is necessary to generate and maintain a breeding population of adult fish, which does require a licence.

Typically, what will be done to an animal used in your project?

The adult zebrafish will be housed in a dedicated aquarium within the department, run by trained staff. We will generate genetically altered zebrafish by introducing modified genetic material at the 1 cell embryo stage and growing these embryos to adulthood. In order to know which fish contain genetic alterations we sometimes need to carry out genetic analysis via fin clipping: e.g. cutting a small portion of the fish's tail fin under general anaesthetic and analyse the genetic code inside this tissue. The fish is then kept in a separate tank with fresh water and the fin then regrows relatively quickly (within approximately 2 weeks). Where appropriate, other methods of genotyping may be used, such as swabbing the surface layer of the skin. Adult fish will be maintained until a maximum of 18 months of age. When possible, healthy fish may be maintained until 30 month of age on a specific ageing protocol to reduce the number of animals used in the project. During this time, adult fish will be bred in specialised breeding tanks to enable the production of genetically altered zebrafish embryos. We very occasionally need to anaesthetise fish for the collection of eggs and sperm. At the end of the protocols fish will be humanely killed or supplied to other project licences or recognised establishments with the authority to breed and maintain genetically altered zebrafish of this type.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not usually expect there to be adverse effects to adults caused by the genetic alterations that we introduce. However, for example in the case of introducing fluorescent reporters for subcellular components, it is sometimes possible that, due to basal activity of these genes, some adverse effects might arise in the fish. If this occurs, we would expect such effects to be mild (such as thinner bodies, mild lordosis that does not affect swimming). However, it is possible that sometimes moderate effects might occasionally arise (such as significantly bent body-axis, which might affect swimming). It is also possible that the survival of larvae to adulthood might not be as high in some genetically modified lines when compared to wild type lines. We do not expect there to be any adverse effects from breeding the zebrafish. It is unlikely but possible that fish might develop an infection following removal of a small part of the tail fin, in which case we will humanely kill the fish. For both genotyping and sperm/egg collection, it is possible that fish may not recover from anaesthesia but this is very unusual (less than 1%).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Zebrafish. Mild: 85%. Moderate: 15%

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The focus of this project is understanding how cells cope with mechanical stresses when squeezing in small spaces inside the body of a living animal. It is important to use an animal rather than looking at cells in culture because the physical properties of cells in a dish and the interactions between cells and their environment are very different in a culture system. Therefore, it is only possible to fully understand the cellular responses to mechanical stresses by looking inside an intact animal. However, we are able to carry out some of our research using cells grown outside an animal (cell culture) – for example we have recently started a collaboration with another team of researchers to understand how human neural crest cells derived from embryonic stem cells cope with being squeezed in a dish and have accordingly reduced the number of animals used in our research. As we investigate the animal model alongside this culture system, we will learn whether more of our work can be carried out in culture.

Which non-animal alternatives did you consider for use in this project?

We have introduced the use of cell culture models in our lab, which we will use in the near future in collaboration with another team of researchers to replace some of our animal experiments. For example, our animal experiments will allow us to measure the size and shape of the spaces where neural crest migrate inside the embryonic animal tissue. We will use microfluidics to generate channels of similar size and shape and investigate how cultured cells respond to compression in these small spaces. We estimate that carrying out this work in culture will reduce our anticipated animal usage by roughly 20%.

Why were they not suitable?

It is not possible to entirely replace animal experiments for this project since it is important to test whether the principles identified in cell culture are the same in vivo. This is especially important since the main aim of this project is understanding the consequences of mechanical stress on migrating and dividing cells in vivo, which will be dependent on the physical properties of the tissues surrounding our cells of interest.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 our experiments will be carried out on embryos younger than 5 days post fertilization (dpf), which are not protected under The Animals (Scientific Procedures) Act 1986.

Animals older than 5 days old will only be used for establishing genetically altered zebrafish for subsequent breeding.

For this reason, the numbers of zebrafish necessary to carry out the research we are proposing will only depend on the number of Zebrafish that are required for breeding. For a robust stock we maintain approximately 2 tanks of 20 fish per transgenic/mutant line with a 50:50 male to female ratio (40 fish/line). For each stock a new generation will be grown every 18 months (3.3 generations per line in 5 years). Because sometimes most fish in a tank will end up being of the same sex and this is difficult to control, it often can result in a gender bias, so we will raise double the number of fish that will be eventually maintained (80/line).

We will maintain 20 existing transgenic lines and two wild type lines and generate ten new transgenic lines (80x32 lines=2560 fish). For each we will breed a new generation every 18 months (2560x3.3=8448). When generating new lines, the F0 embryos will need genotyping: approximately 100 additional fish will be required per new line at the F0 stage to find founders for the F1(100 x 10= 1000) for a total of 2560+8448+1000=12008 fish.

However, we anticipate that the numbers that we will actually use should be significantly lower, since we are committed to actively reducing animal usage (see below).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Animals older than 5 days old will only be used for establishing genetically altered zebrafish for subsequent breeding. Therefore, it is not possible to reduce the number of licenced animals used via experimental design.

However, we still aim to reduce the number of unlicenced zebrafish embryos under 5 days old that we will use in our experiments via careful experimental design. There are several experimental design assistants and guidelines available to help us with appropriate design of each experiment. For example, the NC3Rs EDA (https://www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda) and PREPARE guidelines (https://norecopa.no/more-resources/experimental-design-and-reporting/).

We will also ensure that our publications conform to the ARRIVE guidelines: https://www.nc3rs.org.uk/arrive-guidelines.

For example, to make our experiments robust, we will control for variability in the following ways:

We will reduce environmental variability by carefully housing breeding adult fish in the dedicated zebrafish facility and by keeping genetic background constant within each genetically modified line of fish.

We will assess normal levels of variability within experiments via pilot experiments, allowing us to select appropriate statistical methods and number of embryos.

We will reduce bias by randomly selecting embryos collected from a pool of breeding adults and, when possible, by assigning treatment and control groups in a way that is unknown to the person analysing the data (blinding).

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 several strategies to reduce the numbers of adult animals used. First, we will share relevant fish stocks with other users within the facility. Second, we will try to limit the number of new generations being grown for each line by maintaining fish up to the age of 30 months when possible. Third, we will minimize the generation of transgenic lines and use wild type embryos wherever possible for our experiments. Fourth, we will freeze sperm from genetically altered lines of zebrafish for longer- term storage. We will also carry out efficient genotyping and raise fewer fish per generation wherever possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For this project we will use Zebrafish to establish and maintain breeding stocks of fish that will be used to produce genetically altered embryos. Because the Zebrafish embryo is small and translucent, it is especially suited to investigate how cells dynamically respond to mechanical stresses in an intact animal.

Why can’t you use animals that are less sentient?

The key aspect of our programme is to visualise and perturb neural crest cells behaviours in situ by advanced microscopy techniques or micromanipulation. We use the Zebrafish because it offers unique advantages for imaging studies due to its optical transparency: in contrast with mammalian tissue, Zebrafish embryos can be imaged in a completely non-invasive manner.

Since neural crest are an evolutionary feature of vertebrates, to investigate neural crest cell behaviours it is necessary to use a vertebrate model system. The zebrafish embryo is also an ideal model system because they are small, transparent, develop rapidly and it is possible to alter their genetics in a straight-forward way.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We don’t envisage any suffering in the vast majority of licenced animals beyond the mild procedures described above. Moderate effects might occasionally arise due to the genetic alteration of the fish. If that occurs, the animals will be killed using a humane method as soon as possible. In certain occasions, for example if a fish is the founder of a new transgenic line and develops moderate effects, it will be killed as soon as the next generation reached breeding age and bred successfully, to minimise suffering without hindering the progress of the project. We will only use zebrafish embryos younger than 5 days old for our experiments, which are not yet capable of independent feeding. We will aim to reduce any potential suffering of these embryos by promptly killing them using a humane, approved method at the end of the experiments and by anaesthetising embryos that are sufficiently developed to be capable of initiating movement during imaging (those above 18 hours old).

Adult fish will be housed in a dedicated centralised zebrafish facility, where they will be looked after by full time staff, who will ensure their welfare. Numbers of fish per tank, water quality and food quality and quantity will be carefully controlled. Environmental enrichment is currently provided to the fish by feeding them live invertebrate foods, providing them with a small current of water they can swim against and occasionally by enriching the environment with plastic plants.

Whenever fish may have to be maintained in tanks in small numbers (i.e. when transgenic founder fish are identified) they will be housed with companion fish that can be distinguished by their body pigmentation or be housed in a tank with environmental enrichment.

If a transgenic line is not being regularly used, it will be preserved by sperm freezing to ensure that no unnecessary numbers of animals are generated.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Appropriate experimental design for our experiments in unlicenced embryos under 5 d.p.f. will be carried out, as described in the ‘Reduction’ section above. Licenced animals older than 5 days old will only be used for establishing genetically altered zebrafish for subsequent breeding. These will be housed in a dedicated centralised zebrafish facility, where they will be looked after by full time trained animal technicians, who will ensure their welfare, in line with their training on best practice. We will make use of available resources to inform us about the current research on refinement of procedures (e.g. https://norecopa.no/species/fish/, https://nc3rs.org.uk/3rs-resources/zebrafish-welfare, https://www.lasa.co.uk/current_publications/). These will be taken into account when deciding on the most appropriate method for procedures.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Advances in 3R tools are internally circulated. We can also access advances via the NC3Rs (https://nc3rs.org.uk/resource-hubs) and Norecopa website pages (https://norecopa.no/databases- guidelines). If scientifically appropriate advances in 3Rs arise in the course of the project, we will seek advice from the named veterinary surgeon and named animal care and welfare officer about whether and how to implement them.

86. Neural circuits and cells that underpin somatosensation and pain

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

somatosensation, pain, neurons, circuits, behaviour

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to understand how sensory information is generated by the body, how the nervous system uses this information to shape behaviour, and how these mechanisms are altered in pain states. Using state-of-the-art techniques, unravelling these mechanisms will establish fundamental knowledge and identify potential targets for the treatment of chronic pain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chronic pain affects around 1 in 5 people worldwide and has a devastating impact on individuals, their families and caregivers. Treatments for many forms of chronic pain are generally ineffective, as the underlying mechanisms remain obscure. Neuropathic pain is one example of this - it results from the dysfunction of the nervous system and affects 7-

8% of people (Colloca et al., 2017). Major causes of neuropathic pain include diabetes, shingles, multiple sclerosis, stroke, and cancer as well as common conditions, such as radiculopathies and traumatic or postsurgical nerve injuries (Colloca et al., 2017). Our research addresses the operation of the somatosensory system and how this is altered in pain states. The intention is that addressing these fundamental mechanisms will directly inform the development of much needed treatments for chronic pain states. The use of advanced tools to understand these processes will enhance the knowledge base across a wide range of fields (neurobiology, physiology, pharmacology, genetics, anatomy, bioengineering, and chemical biology), but will ultimately aim to benefit the health of individuals and our society.

What outputs do you think you will see at the end of this project?

Our research will address how the nervous system encodes information and how this process is transformed in pain states. We will provide new fundamental knowledge of somatosensory and pain mechanisms that will be published in open-access journals. This research aims to lay the foundations for new treatments for human chronic pain.

Who or what will benefit from these outputs, and how?

Chronic pain affects more than 1.5 billion people worldwide and can be highly debilitating, impacting quality of life, society, and the economy. This is estimated to cost the United Kingdom £1.6 billion per year. Chronic pain significantly reduces the quality of life of individuals, their families, caregivers, and places a large burden on the healthcare system, which is likely to worsen with an aging population.

In the short-term, the outputs from this project will expand the knowledge of the fundamental somatosensory and pain mechanisms and will be disseminated to the research community at meetings and papers in a timely manner. In the longer term, the intention is that addressing these fundamental mechanisms will inform the development of much needed treatments for chronic pain.

How will you look to maximise the outputs of this work?

The timely communication of the knowledge arising from this research will be important to ensure that they achieve their maximum impact. Results obtained from this work will be shared with scientific and medical communities through conferences, seminars and consortia, and disseminated through publication in open-access international scientific journals that reach a wide audience.

Species and numbers of animals expected to be used

- Mice: 9000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are selected as the simplest animal model that can most readily provide insights into how the brain processes somatosensation and pain. Mice are the primary animal model for mechanistic studies of the brain due to the wide variety of techniques for recording neural activity, diverse transgenic and viral methods that enable causal manipulations, and well-established experimental paradigms for examining behaviour. We will investigate the neural basis of somatosensation and pain in adult mice.

Typically, what will be done to an animal used in your project?

The project will establish how the nervous system uses somatosensory inputs to shape behaviour, and how these mechanisms are altered by pain states. Typically, adult mice will undergo surgery to enable manipulation of the nervous system by stereotaxic injection of genetic tools or recordings of the nervous system by implantation of miniaturised recording devices. Manipulation and recordings will be made during behavioural assessment - motor and somatosensory function will be assessed, along with spontaneous, exploratory, and goal-directed behaviours. Models of pain states will be used to examine changes in behaviour and neural activity, and conversely, how neural activity changes pain states. The objective is to discover neural circuits and cells that underpin somatosensation and can effectively reduce pain states.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will be exposed to mild and transient pain and models of persistent pain that aim to replicate human chronic pain conditions. Mice are closely monitored throughout experiments, and we have extensive experience of maintaining high health status by animal husbandry. Close monitoring and husbandry ensures that any potential clinical signs, such as weight loss or abnormal behaviours, are quickly detected, controlled and limited. Experiments are never continued longer than is absolutely necessary.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Breeding and maintainence (~20% of mice) is mild while our surgeries, pain state models, and recordings/manipulations are moderate. Many mice (~80%) in this project have the potential to approach moderate severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

During this project, we will investigate how the somatosensory system guides specific functions, drives pain, and is altered by pain states. This system is highly interconnected. Sensory receptors in our skin detect specific stimuli and send information to intricate networks within our spinal cord and brain that play a pivotal role in determining our actions and behaviour. There are no computer simulations, in vitro approaches, or equivalent that can accurately and effectively model these phenomena and so we require that animals are used in these experiments.

Mouse models offer the ability to produce a fine-tuned characterisation of behaviour and the roles of specific cells and circuits, alongside the benefits of excellent temporal and spatial resolution with imaging and electrophysiological recordings of cellular activity. The data obtained during this project will allow for more precise understanding of mechanisms underpinning somatosensation and pain, which represents one of the biggest clinical challenges to date.

Which non-animal alternatives did you consider for use in this project?

We have considered using computer models and cultured cell lines as a non-animal alternative.

Why were they not suitable?

Computer modelling requires an accurate understanding of rules and logic of the system being modelled. There is currently an incomplete understanding of the neural computations that are carried out in somatosensory and pain circuits. Thus, there are no computer models or equivalent that can model these phenomena, and so experiments on whole animals are required.

No cultured cell lines are available to study the natural circuit mechanisms, and so intact tissues must be used.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated numbers of animals are based on the research goals of my laboratory under this license over the next 5 years. Appropriate experimental designs – unbiased and adequately powered studies – are applied in order to identify the minimum number of animals we need to use and identify the best technical approaches we can employ to answer the specific question being posed. We have extensive experience in animal somatosensory and pain behavioural studies and have found that for the paradigms described in Protocols 2-5 we can obtain statistically meaningful data with groups of 5-12 animals (see also Mogil JS et al., 2006. Pain 126, 24).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The studies we intend to conduct will be designed so as to minimise the number of animals used to demonstrate a given statistically significant effect. Generally, we run pilot studies (2-4 animals per condition) to explore the parameters to determine final experimental design. The NC3Rs Experimental Design Assistant is used to aid this process. We may obtain additional advice from statisticians when required.

At each stage the same animals will be tested in multiple paradigms to minimise numbers used, and to maximise the data collected. For example, multiple electrophysiology or optical recordings can be achieved from one animal.

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 optimise our breeding strategies to produce minimal numbers of animals. For example, we will breed homozygous floxed mice with mice that express one copy of a Cre recombinase, so that all the progeny of the mating will either be experimental animals (i.e. floxed genes expressed) or appropriate controls (i.e. floxed genes will be present but not expressed). Both male and female mice are used.

We have chosen a range of models to cover different types and pathologies of pain as it is clear from previous research that there is not one common pathway for all types of pain. The number of different models allows us to look specifically at a pain pathology (i.e. allodynia caused by neuropathy) and reach our objectives efficiently and by using as few animals as possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used for this project as they represent the simplest species appropriate for this type of work. We apply many well-established methods that have been refined over decades to establish the mechanisms of somatosensation and pain. Animal suffering will be limited in our studies by our strict monitoring of animals with regards to severity limits of associated protocols, and our protocols have been carefully designed to minimise trauma and suffering. Experiments will not be continued for longer than is absolutely necessary.

Why can’t you use animals that are less sentient?

Mice are the least sentient animal model to study the how the mammalian brain processes touch and pain. Non-mammalian brains cannot be used for forward and reverse translation as effectively due to anatomical and functional dissimilarities. Less sentient or anaesthetised mice would not allow us to address our scientific questions. The nervous system has not developed sufficiently in neonatal and juvenile mice and adult mice must be used. The mouse is also highly genetically tractable, allowing transgenic identification

of specific cell types crucial to the fulfilment of the project. There are highly advanced and efficient techniques developed for the mouse as opposed to other mammalian species. For example, there are excellent stereotaxic maps of the mouse brain, allowing accurate targeting of specific brain regions.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The administration of analgesics may potentially interfere with the measured variables of our studies. We will minimise any potential suffering by administering pain relief after surgery in cases where this does not itself compromise the validity of pain models. Following all chronic pain models, we will monitor the animals for potential pain, suffering, distress, and any unexpected behaviours, observing the severity limits of the protocol. The animals will be culled immediately after the last time point of testing or as soon as social behaviour, grooming, weight loss/gain and/or wound healing indicate the animal is suffering according to severity monitoring sheets which will be kept in the animal holding room throughout the experiments. Mice will receive environmental enrichment and are group housed whenever possible and compatible with the experiment.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We ensure experiments are carried out in accordance with the 3Rs and the ARRIVE guidelines. We will apply a Culture of Care (norecopa.co). Surgery will be carried out according to aseptic techniques and LASA guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We stay informed about recent developments in the 3R through the NC3Rs website, through technological advances published in international journals (e.g. Nature Methods), and through dialogue with the BSU staff and NVS, to ensure the most refined models and methods are used. We implement these advances to our experiments where appropriate - for example our custom analysis pipelines leverage recent developments in machine learning, reducing observer and experimenter bias to improve reproducibility while increasing the sensitivity of our measures to reduce the number of mice needed.

87. Development and Adaptability in Central Nervous System Sensory 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
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

pain, touch, movement, critical period, plasticity

Animal types	Life stages
Mice	aged, 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?

We wish to understand the postnatal development of integrated touch and pain processing and behaviour and how these are affected by early life experience.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Each element of the programme will lead to an increase in the basic scientific knowledge of infant and childhood pain. Furthermore, it will provide a scientific rationale for the design of better analgesic management and help us to understand the long term consequences of pain in children. Through our extensive clinical collaborations, we are able to apply our scientific knowledge directly to the clinical setting.

What outputs do you think you will see at the end of this project?

The scientific outputs of this research will be:

- Understanding the structural and functional plasticity of excitatory and inhibitory neurons responsible for processing pain in infant spinal pain circuits.

- Mapping the postnatal development of neuroimmune interactions in spinal and cortical pain circuits in response to early life tissue and nerve injury.

- Discovering how the maturation of connections and neurotransmitter in specific brain nuclei affects the descending control of spinal pain circuits

- Structural and functional analysis of pain or potentially painful activity in the infant cerebral cortex.

Who or what will benefit from these outputs, and how?

The benefits of this research will be:

- Scientific knowledge of the spinal circuits that integrate infant pain behaviour.

- Understanding how the brain can control spinal pain processing in early life

- A full picture of the role of neuroimmune interactions in early life pain circuits

- A scientific basis for developing new analgesic strategies in animal models of childhood pain

- Measurement and potential prevention of the long-term effect of repeated tissue injury upon the developing CNS.

- Exploration of the neural basis of the perception of pain by the infant cortex.

When the benefits will be delivered

- The increase in scientific knowledge of mechanisms underlying infant and childhood pain will be immediate

- Within the life of this project we will also change clinical perception and inform approach to treatment.

Within and beyond the life of this project we will directly influence paediatric pain treatment. Our animal based pain research has led directly to translational work with clinical colleagues, funded by the Wellcome Trust and the Medical Research Council, using new neurophysiological measurement techniques to measure CNS pain processing in hospitalised infants.

How will you look to maximise the outputs of this work?

We regularly present at both international and national conferences and currently have international collaborators We are also passionate about science communication and interacting with the general public to disseminate ideas

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.

The study of touch, pain, itch and movement has been extensively studied in the rodent. Mice in particular provide the advantage of both unique and precise genetic access and a broad range of sophisticated quantifiable behaviours. They are therefore the ideal candidates for the study of senses, results of which can be directly translatable to human physiology and biological function. Mice will be of ages 0-8 weeks and provide an excellent model of human development, corresponding approximately to ages 32 week gestation to adult in humans.

Typically, what will be done to an animal used in your project?

Typically, transgenic animals will receive a subcutaneous injection after birth to allow minimally invasive manipulation of the circuit during early life. Once they reach adulthood, animals will then be tested for motor coordination, adaptation and regularity to assess the role of experience on development of adaptive motor behaviours.

What are the expected impacts and/or adverse effects for the animals during your project?

Injections are minimally invasive and acute and animals recover within a few hours. As a result of manipulations, animals may experience changes in touch, pain and proprioception within the first hour. The manipulations may have long lasting effects on motor behaviour, including loss of balance and misstepping.

Expected severity categories and the 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: 35%

Moderate: 65%

Severe: 0%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of intact animals is critical for this study, as it becomes more apparent that touch and pain are not located to one area of the central nervous system, but rather are distributed. The study of nociceptive circuits has been extensively studied in the rodent. Mice in particular provide the advantage of both unique and precise genetic access and a broad range of sophisticated quantifiable behaviours. They are therefore the ideal candidates for the study of pain, results of which can be directly translatable to human physiology and biological function.

Which non-animal alternatives did you consider for use in this project?

We considered the use of lower vertebrate animals such as zebrafish and of invertebrate animals such as flies and worms for this project due to their genetic tractability and faster gestation. This is an avenue we actively consider and are monitoring research to ensure we are using the most up to date systems with the lowest impact on harm. The use of isolated tissue was considered as an adjunct.

Why were they not suitable?

While other approaches might complement the use of animal models in this project, animal models are wholly necessary and fundamental to understanding how pain develops and its long-term trajectory.

This is in large part due to the complex behaviours that can be examined in the mouse versus that in lower vertebrates and invertebrates and the physiological similarities between mammals. If we are to begin to decipher the nature of pain and long term disease in man this must be in an animal with similar biological circuits. Isolated tissue is similarly not suitable in this study as we are interested in the widespread processing of touch and pain across the body.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We do not use more animals than necessary because it is ethically unacceptable. It is also inefficient and wastes staff time and money. Experiments are designed to minimise animal numbers. In most cases we are measuring the difference between means of dorsal horn spike activity or sensory thresholds, in control and experimental animals. Our data base of

control data combined with pilot studies in 3 experimental animals provides us with the standard deviations required to calculate the required sample size for a significant effect at the 5% level. In practice, in most of our experiments 6-8 control animals and 6-8 experimental animals are sufficient to produce significant data. Dorsal horn electrophysiological data is usually based on 50 cells recorded from approximately 10 animals, behavioural studies from 6 animals per experimental variable and immunohistochemical studies often require tissue from 3 animals per antibody. The animal numbers take into account the fact that for developmental studies each experiment is performed at postnatal day 3, 10, 21 and 40 and that we have a number of different experimental conditions. In some cases, such as in the case of viral injections, control virus injection is necessary because it is essential to know whether the outcome is due to the virus itself or to the preceding surgical procedure. Without it, the whole experiment would be wasted and the data uninterpretable.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have addressed reduction by

1. By taking statistical advice from Statistical Support Services offered locally to check the efficiency of our experimental designs.

2. By reducing the number of time points studied.

3. By introducing longitudinal telemetric recording in single animals to reduce the number of animals used.

4. By undertaking pilot studies in order to perform accurate power calculations for each experiment.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We are committed to the principles of the 3 Rs and always run our experiments so that statistical significance can be achieved with the fewest animals and least harm. This includes running pilot experiments to accurately calculate sample size and using animals as their own internal controls wherever possible (such as using ipsi/contra comparison of tissue and looking at animals pre and post manipulation). We have a rigorous breeding strategy (NC3Rs breeding strategy) to ensure that all transgenic lines are kept efficiently and with minimal waste. We are also part of an Animal Tissue Exchange that ensures that all animals benefit scientific progress.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Pain models are used in this programme of work. The rationale for using them and how we ensure that they are the least severe that will produce satisfactory results is given in the table below. They are performed under general anaesthesia followed by recovery. In all cases, animals will normally be maintained for up to 30 days except where the effect of neonatal interventions upon the maturation of the brain is being studied, in which case it will be for up to 90 days.

These models normally include administration of a local agent for immediate postoperative analgesia. As we are studying the mechanisms underlying pain and these mechanisms may be masked by analgesia, on some occasions analgesics will not administered. However, if the animal shows signs of discomfort the animal will be humanely killed.

Hindpaw or ankle joint inflammation: complete Freunds adjuvant: Local redness & swelling. No spontaneous pain behaviours are observed but mechanical sensitivity & thermal hyper-algesia begins after 24 hrs. A model of lasting allodynia and hyperalgesia by prolonged tissue or ankle inflammation as in juvenile rheumatoid arthritis. No signs of ongoing pain, but mechanical and thermal allodynia on stimulation. The lowest concentration and volume of Freunds adjuvant is used that will produce mechanical allodynia. This model will not be used except where a specific arthritic model is required.

Peripheral hindlimb nerve ligation or section: No overt pain behaviour, lack of sensation in denervated region. Onset of mild allodynia in 24 hours. Lasts for 8 weeks. A model of neuropathic pain as occurs in man following nerve injury. A defined nerve lesion, separating the nerve from its target tissue with little or no regeneration that is consistent across ages and species. Different nerves are used, depending upon their site of innervation and their sensory/motor/autonomic nerve content. Section of one or two peripheral nerves causes no ongoing pain and minimal motor impairment. The mechanical allodynia that develops is the least severe of all the reported neuropathic pain models3.

Local or short acting systemic, such as buprenorphine or meloxicam will be used for post-operative recovery.

Behavioural tests are undertaken so that we can measure any change in sensory thresholds in animals following a particular intervention such as a tissue injury or a CNS injection. Human infants display a ‘tenderness’ or hyperalgesia following tissue injury and this can also be measured in rodents as a fall in the response threshold to mechanical or thermal stimulation of the skin. Motor and open field tests informed us about the state of the animals development and normal range of exploratory activity.

These tests are of mild severity and low stress as the animal is not restrained and can freely escape from the stimulus. They are regularly handled and familiarised with the equipment before testing. It should be borne in mind that human infants in intensive care are exposed to repeated sensory stimulation.

In these experiments, the direct effects upon clinical pain are measured with the ultimate aim of developing better analgesic strategies.

Electrophysiological recordings and optogenetic stimulation are performed to measure the effects of pain models upon neural activity in the spinal cord and brain. Recordings and stimulation are either performed under non-recovery anaesthesia (AC) or in awake animals where electrodes or optodes have been implanted in the brain under anaesthetic with recovery (AB). Electrode/optode placement is undertaken under aseptic conditions and postoperative analgesia applied. This procedure has been found to be well-tolerated with minimal animal distress or mortality. The use of telemetric recording techniques allows animals to stay in their litters and/or home cage as they are not restrained or tethered during recording.

Why can’t you use animals that are less sentient?

Pain experience cannot occur at the cellular or molecular level – it requires an intact nervous system. To understand and prevent pain we need to investigate the changes in synaptic processing and signalling mechanisms that occur at many levels of the intact CNS following tissue injury. This can only be done in animal models and the use of animals is justified by the potential reduction in suffering for infants and children. Detailed analysis of cellular interactions can be undertaken in isolated slices of spinal cord or brain maintained in vitro, but the results need to be interpreted in the light of those obtained from in vivo work as the conditions of study are inevitably less physiological and hence the results could be misleading. Although an interesting genetic model, the use of zebrafish or C.Elegans for the study of pain cannot be justified when approaching translationally relevant studies such as those proposed here due to their lack of circuit homology with the mammalian pain network. Zebrafish in particular do not express the same nerves as are found in both the rodent and in man, rendering them an insufficient pain model.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will regularly monitor animals if they undergo any procedure to look for changes in behaviour, grooming or feeding. Animals will be given post-operative analgesia and pain monitored throughout. To minimise stress during behavioural experiments, they will be habituated to the room prior to testing.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs and RSPCA websites and 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 up to date with any developments on the NC3Rs website and with workshops offered by the NC3Rs and RSPCA. All lab members will be strongly encouraged to attend workshops and seminars.

88. Identifying zoonotic flavivirus emergence pathways and seroprevalence in wild birds in the United Kingdom

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

Flaviviruses, Wild birds, Seroprevalence, Surveilance, emergence

Animal types	Life stages
Blackbird (Turdus merula)	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 identify emergence pathways and seroprevalence data in the UK for exotic, mosquito-borne zoonotic flaviviruses, where wild-birds are the primary host.
Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Flaviviruses are an important group of viruses that can be transmitted by mosquitoes, ticks and sandflies. Many arthropod-borne flaviviruses are zoonotic and the primary hosts are birds. The UK was considered free of mosquito-borne viral zoonoses, until Usutu virus (USUV) was detected in Greater London in 2020 in avian hosts and mosquito vectors. USUV is mildly pathogenic in humans and can cause neurological disease in immunocompromised individuals (to date there have been no USUV infections recorded in

humans in the UK). However, USUV can severely impact passerine birds, especially blackbirds (Turdus merula), infection in some European regions has been sufficient to cause disease mediated population declines. Indeed, following detection of USUV in the UK a reduction in blackbird reporting rates and breeding populations was recorded in Greater London during 2020, the index site for the USUV outbreak. Subsequent detections of USUV in 2021, 2022 and 2023 suggest that the virus is overwintering in the UK and that other zoonotic flaviviruses with similar environmental requirements, such as West Nile virus (WNV) could emerge here and cause disease. WNV has been steadily migrating northwards through mainland Europe and has been detected in Germany and the Netherlands in recent years through live wild bird surveillance (using swabs, serology and feather sampling, which are not routinely carried out at the moment in the UK). WNV potentially has a greater impact on animal and human health when compared to USUV and is a notifiable disease of horses in the UK.

Current surveillance for avian zoonotic flaviviruses requires submission of recently deceased wild birds for post-mortem examination through regional organisation laboratories combined with a national surveillance programme that focuses on garden birds (www.gardenwildlifehealth.org). While this passive surveillance approach is appropriate to detect mortality caused by viral disease; the species complement is biased towards synanthropic species (with infrequent migratory bird submissions); the convenience sample obtained does not meet random sampling assumptions and therefore cannot be used to calculate prevalence estimates; and no active surveillance with sampling from live birds is currently undertaken. Carcass submission cannot provide inference on seroprevalence in populations as sera can’t be obtained from dead birds, nor is it useful for understanding emergence pathways, as migratory bird carcasses are infrequently found and submitted for testing. Therefore, we applied for funding to enhance surveillance for zoonotic avian flaviviruses to identify emergence pathways and seroprevalence data through migratory and native bird sampling. This information will identify avian based emergence pathways for zoonotic flaviviruses and provide seroprevalence data for a suite of primary avian host species. This study will generate data that can be shared with a UK based vector- borne disease hub, relevant organisations and stakeholders and be used to inform policy to protect UK biosecurity. Alongside the sampling of wild birds detailed in this licence application, geographically associated mosquito populations would also be sampled (through the funded grant) to check for virus presence in vector populations. The mosquito screening does not require a licence, but it enhances the work we are undertaking in this licence to help build a more detailed picture of flavivirus epidemiology in the UK. Data generated from both streams (wild bird and mosquito sampling) would be combined in any reports to maximise their impact and improve the benefits of undertaking the licenced work.

What outputs do you think you will see at the end of this project?

Analysis of avian specific emergence pathways for a range of zoonotic flaviviruses, with a focus on Usutu virus and West Nile virus, including seroprevalence data for a suite of UK wild birds that are primary host species.

Improved detection of flaviviruses in birds using cloacal and buccal swabs and feather sampling, combined with serology to evaluate historical virus exposure, which will improve current government surveillance activities and outputs.
Data to inform policy and safeguard UK biosecurity.

Enhanced collaborative network between organisations to investigate vector-borne disease.

Collaborative, multidisciplinary publications on flavivirus emergence and transmission in a temperate region.

Further dissemination of results from this project will be through:
National Reference Laboratories and the Garden Wildlife Health Project/ links to the British Trust for Ornithology and Royal Society for the Protection of Birds/ Human Animal Infections and Risk Surveillance group/ UK HSA Emerging Infections and Zoonosis group.

Who or what will benefit from these outputs, and how?

Researchers, organisations, policy makers and relevant stakeholders interested in viral emergence and transmission pathways. This benefit will be apparent upon open access publication of data via the UKRI funded vector-borne disease hub, peer-reviewed publications and through organisation chains of communication. Open access data will made available within three years following completion of the project.

The flavivirus surveillance programme, through enhanced surveillance activities and outbreak preparedness. New sample streams will be generated increasing our ability to detect flaviviruses in wild birds. This benefit will be realised throughout the project as sample volume increases.

Organisations and stakeholders involved in mosquito surveillance and control programmes as detection of flaviviruses will result in targeted mosquito surveillance and could be used to inform delivery of vector population control programmes to safeguard UK biosecurity.

How will you look to maximise the outputs of this work?

The findings from this study will be published it is likely that more than one publication will arise as the study will look at a multifaceted approach to understanding flavivirus emergence and transmission pathways alongside seroprevalence data in wild birds. This paradigm maximises research outputs and therefore wider scientific community understanding, while avoiding the need for duplicated efforts in this area and by extension reducing animals required for such detailed outputs.

Shared knowledge with collaborators from mainland Europe and stakeholders in understanding flavivirus phylogeography and emergence patterns in temperate regions. Following publication of findings from this investigation we would also disseminate this knowledge at national and international conferences in the hope that it maximises its potential target audience and can assist in understanding flavivirus impact globally.

Our multidisciplinary team includes representatives from organisations who would report and engage with the general public to increase awareness of flavivirus-associated disease in UK wild birds and humans.

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.

Passerine birds are a primary host for a number of zoonotic flaviviruses. In some species of bird, such as blackbird (Turdus merula), the impact of USUV infection has been sufficient to cause disease mediated population declines. Avian hosts are a key indicator species used across mainland Europe to undertake surveillance for zoonotic flaviviruses and detection in birds typically precedes detection/infection in humans, so it can act as an early warning system to prepare public health messages and responses. Consequently, enhancing surveillance activities, by including serology, feather sampling and swab-based surveillance in a number of key migrant and native wild birds we will mirror practices in mainland Europe which have been successful in detecting the geographic expansion of mosquito-borne viral zoonoses. Undertaking surveillance in avian hosts represents the only option for understanding how avian zoonotic flaviviruses, such as USUV, have emerged in the UK, and will highlight at risk geographic areas that may warrant targeted mosquito control programmes. As this is opportunistic surveillance, the life stage of a given bird will depend on what is caught for sampling on a given day and therefore cannot be controlled for in our surveillance paradigm; consequently, we propose to sample both adult and juvenile birds. The focal species for targeted surveillance are Blackbird (Turdus merula), Blackcap (Sylvia atricapilla), House sparrow (Passer domesticus), Common whitethroat (Curruca communis) Lesser whitethroat (Curruca curruca) and Willow warbler (Phylloscopus trochilus) as these represent the likely migrant and native birds that are susceptible to flavivirus infection and/or associated disease. By restricting our sampling to a subset of primary host animals we believe this will reduce the number of animals that need to be sampled while simultaneously providing robust data on flavivirus prevalence and emergence pathways. All procedures defined in this application would be undertaken by trained and competent staff or volunteers who are licenced to catch and handle birds.

Typically, what will be done to an animal used in your project?

Each sampled bird, following capture (capture is a pre-existing process that is undertaken by trained and licenced ringers or observatory staff using a mist net or bird traps [both <1% mortality- British Trust for Ornithology (BTO) personal comms]), initial health assessments would be undertaken by trained and Natural England (NE) licenced BTO staff or volunteers and would not be undertaken by the personal licence holders covered under this licence application, this collaborative work has been agreed with BTO. During initial health assessment the bird would be weighed (a pre-existing practice for monitoring wild birds and carried out by trained BTO staff or volunteers) to ensure they meet the required weight for blood sampling. The minimum acceptable weight for collecting a blood sample (either by needle draw or capillary action) is 18.2g to ensure 100µl can be obtained for downstream analysis [bird weight ranges data courtesy of BTO, blackbird 86.0-122.0g, blackcap 15.4-21.0g, house sparrow 22.8-30.7g. common whitethroat 12.0-17.6g, lesser whitethroat 10.2-13.5g, willow warbler 7.4- 10.4g, therefore blood sampling (by needle draw or capillary action) common whitethroat, lesser whitethroat and willow warbler is highly unlikely due to known weight ranges]. However, for birds under our weight threshold of 18.2g we will take a single blood spot, following vein puncture, of no more

than 20µl on a protein preservation card to attempt to detect flavivirus specific antibodies. Only once a bird has been deemed acceptable for sampling based on an initial health assessment by BTO staff or volunteers will they be handed to the personal licence holders to perform the procedures detailed below. Each bird will be subjected to cloacal and pharyngeal swab sampling and contour feather removal to detect any active flavivirus infection. Following this a single blood sample (minimum volume 100µl; no more than 10% of circulating blood volume (www.nc3rs.org.uk/3rs-resources/blood- sampling/blood-sampling-general-principles)- which is sufficient in birds that weigh more than 18.2g to enable us to undertake both broad spectrum (pan-flavivirus) ELISA (required blood volume 25µl) and antibody specific PRNT (required serum volume 25µl) assays) will be collected from either the right jugular or ulnar/basilic veins to detect anti-flavivirus antibodies to inform seroprevalence in birds where this is deemed safe (based on body weight calculation) and possible (based on practicalities of sampling- not all birds will yield a blood sample and that is a consequence of the small size of birds we are screening). Any bird that cannot be blood sampled (using a needle draw or capillary action) due to not meeting our weight threshold of 18.2g, will have no more than 20µl of blood removed following vein puncture (this volume falls well within acceptable limits of removal on smaller birds and provides a mechanism by which to detect flavivirus antibodies) from either the right jugular or ulnar/basilic veins for protein preservation card analysis. By combining swab, feather and blood samples we will be able to gain understanding in the seroprevalence of birds arriving in the UK, and also to detect virus which is essential for understanding transmission routes and epidemiology. While swab sampling can indicate presence of a virus, the process may be susceptible, however unlikely, to contamination which would not facilitate inferences on active infection, by combing with feather collection we would be able to screen feather pulp which would confirm active infection and provides material for attempts at virus isolation using Vero cells. Moreover, we will appraise the utility of contour feathers to detect flaviviruses, which we hope can be utilised as a less invasive surveillance tool for the future. No schedule 1 procedures will be carried out. Once the described procedures have been completed by personal licence holders the bird would be returned to the trained and licenced staff or volunteers for a final health assessment, if the bird is deemed fit for release it is returned back into the wild.

What are the expected impacts and/or adverse effects for the animals during your project?

Injury during capture <0.5% (mainly slight abrasions) Mild post sampling haemorrhage <1%

Any adverse effects will be recognised visually as a trained licence holder attached to this proposal will be observing the bird throughout the process from capture to release. In the rare event of haemorrhage, the handler will move the bird to a quiet area to minimise stress and direct pressure on the venepuncture site until haemostasis is confirmed, or surgical glue will be used to seal the wound.

Following stemming of haemorrhage, a bird will be constantly observed to ensure no further veterinary intervention is required, such as subcutaneous fluid administration, before being released into the wild by trained and licenced staff or volunteer ringers. Previous experience of blood sampling in wild birds undertaken by the organisation has found no long- term welfare effects, and short-term effects are most likely related to the stress of being handled rather than procedure, so it is assumed that only mild discomfort is expected.

When swabbing birds an appropriate sized moistened (using molecular grade H20) swab for body size will be used and the PIL holder will monitor the animal for signs of stress/discomfort. A maximum of six contour feathers are to be removed for virus detection (We have shown that feather pulp from these feathers is sufficient to detect virus in infected birds- pilot data using USUV infected blackbirds from the UK). Again, any short-term effects of swabbing and feather removal are linked to the stress of being handled rather than the procedure so only mild discomfort 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)?

Any sampled animal will not undergo anaesthesia so there will be mild discomfort from blood sampling, swab and contour feather sampling. However, most short-term stress, including increased body temperature and increased heart rate are primarily linked to the capture and handling procedures. Only mild-discomfort is expected as such the impact of any procedures is deemed to be low.

What will happen to animals at the end of this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are aiming to capture real-world data on seroprevalence and emergence pathways for zoonotic flaviviruses where birds are the primary host. Consequently, the screening of wild birds is essential to obtain the required data. This project has been funded by UKRI for three years and follows a similar approach to flavivirus surveillance projects in mainland Europe which screen wild birds through serology and swabs to detect the presence and transmission of zoonotic flaviviruses. There are no alternative, non-animal, sampling protocols which could provide the same data. In addition, as this will be the only project undertaking this work in the UK we have taken care to avoid duplication of effort.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives to obtain the required data which will inform policy and safeguard UK biosecurity. Dead birds are currently sampled, and this passive surveillance network will continue and findings will complement data collected in this study; however the species complement is restricted, the sample non-random and collection of sera is not possible, and therefore passive surveillance is not sufficient to inform our understanding on emergence pathways and seroprevalence of flaviviruses where the main host are birds. We have mosquito and cell culture models available, but these can only inform infection of vector and viral tropism post infection. Consequently, live birds, the primary hosts, are required for this licence to elucidate how zoonotic viruses are emerging in temperate areas, such as the UK, and to inform seroprevalence. This understanding can

help inform public health messages and targeted mosquito control measures to help mitigate the transmission and impact of zoonotic mosquito-borne viruses in the UK.

Why were they not suitable?

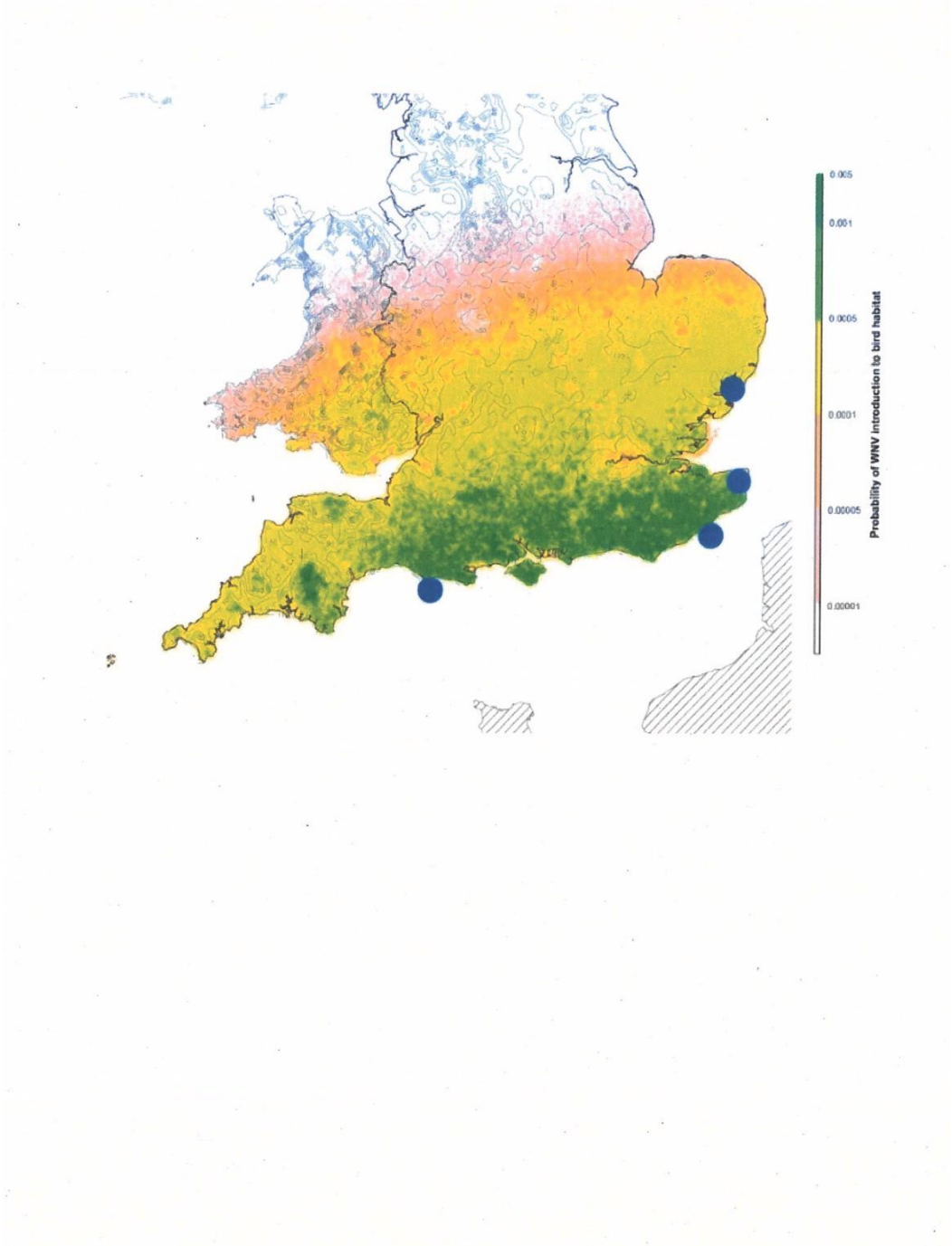
As above, only live wild birds would be able to provide the data required for the project. Cell culture or mosquito models would not be able to provide data on real-world prevalence and emergence pathways for zoonotic avian flaviviruses.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 consulted with statisticians to ensure our sample size is sufficient to be able to detect seroprevalence and reasonably provide an opportunity to detect emergence of flaviviruses not currently present in the UK, in migrant and native wild birds. Using a power level of 0.8 and a 95% significance level 3500 birds are required to detect WNV RNA, based on the current detection prevalence of WNV in wild birds in the Netherlands from 2020 (n= 2783 birds sampled = 1 positive for WNV RNA),as WNV has not been detected in the UK; 108 birds are required to be sampled to detect USUV RNA, we would note that this sample size is based on UK data from current passive surveillance data for passerines 2020-2023 inclusive. Finally, 280 birds are required to detect USUV antibodies, based on seroprevalence data from the Netherlands in 2018 (n= 265 birds sampled = 7 seropositive, USUV seroprevalence work in wild birds has not been undertaken in the UK to date). Consequently, our study seeks to generate pilot data on flavivirus seroprevalence and virus prevalence in birds which can be used to inform more robust power analyses for UK specific sampling paradigms. To increase the likelihood of successful detection and to reduce the number of animals that are required to be sampled our surveillance while obtaining the most epidemiologically relevant data for the viruses our sampling sites are targeted geographically towards areas deemed at high risk of viral incursion in southern England and include the region where the USUV index site is located in Greater London (figure 1).Figure 1.



Risk map for the South of England, showing risk of West Nile virus (WNV) introduction during a spring bird migration season from France (map courtesy of Bessel et al. 2014). Isopleths represent lines of the mean number of days with an average temperature above 14oC. Green areas are those considered most at risk of WNV incursion, blue dots indicate our proposed observatory sampling points, stars are confirmed Usutu virus (USUV) detections. Targeted ringer stations for sampling will be located across Southern England.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The calculated target sample size will facilitate insights into the geographic expansion and seroprevalence of USUV in a range of migrant and native wild birds in the UK. However, the practicalities of achieving our maximum sample size means we are unlikely to have

sufficient power to be able to detect the emergence of WNV in UK wild birds. This virus has not been detected in the UK but its emergence in the UK is becoming increasingly feasible, especially following its detection in the Netherlands in 2020 in a common whitethroat.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

This project will maximise samples taken per animal to avoid duplication of effort or the requirement for additional animal sampling. Each animal, where possible (based on weight and practicalities of sampling) will be sampled for antibody presence using serology and detection of virus using feather sampling and cloacal and pharyngeal swabs.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 focal species for targeted surveillance are Blackbird (Turdus merula), Blackcap (Sylvia atricapilla), House sparrow (Passer domesticus), Common whitethroat (Curruca communis) Lesser whitethroat (Curruca curruca) and Willow warbler (Phylloscopus trochilus) as these migrant and native wild bird species are considered likely to be at comparatively high risk of flavivirus infection and/or associated disease based on wild bird surveillance findings from Europe to date.

All catching and handling of target birds for the procedures is conducted as per British Trust for Ornithology (BTO) guidelines, by handlers who are deemed competent by a BTO license holder and under sufficient supervision by a BTO license holder. The ringing stations and bird observatories in our surveillance network have staff and volunteers that are proficient in wild bird capture and handling which will help minimise stress associated with catching.

Where possible, birds will be sampled when they are already being caught for other purposes (eg. during biannual census or when rings are being added or checked). Consequently, this will reduce the number of necessary catches and by extension reduce stress caused to any wild birds. Handling time will be kept to a minimum.
All procedures post capture, will be carried out by a trained and competent PIL holder to minimise the stress and associated impacts of sampling to any animals.

Why can’t you use animals that are less sentient?

We are aiming to capture real-world data on seroprevalence and emergence pathways for zoonotic flaviviruses where birds are the primary host. Consequently, the screening of wild

birds is essential to obtain the required data. There are no alternative life-stages or less sentient animals that would provide the same data into flavivirus emergence and seroprevalence.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All catching and handling of target birds for the procedures is conducted as per British Trust for Ornithology (BTO) guidelines, by handlers who are deemed competent by a BTO Natural England license holder and under sufficient supervision by a BTO license holder. The ringing stations and bird observatories in our surveillance network have staff and volunteers that are proficient in wild bird capture and handling which will help minimise stress associated with catching.

Where possible, birds will be sampled when they are already being caught for other purposes (e.g., during biannual census or when rings are being added or checked). Consequently, this will reduce the number of necessary catches and by extension reduce stress caused to any wild birds. Handling time will be kept to a minimum.

All animals will be observed by a trained PIL holder linked to this proposal following any procedure to ensure there are no adverse effects of sampling before being released back into the wild. There is no evidence that our prescribed procedures will have any long-term impact.

An assessment will be made by a suitable trained and licenced person who is experienced in handling and releasing wild passerine birds. If no adverse effects to the animal’s state of health occurred, they will be released back to the wild by licenced BTO staff or volunteers. In the unlikely event of any adverse effects the Named Veterinary Surgeon will be called, and their advice taken on whether mitigating procedures such as surgical glue or subcutaneous fluid could be used to improve the condition of the bird- to be undertaken by MRCVS PIL holder only. In the unlikely event that euthanasia was required this would be undertaken using a schedule 1 procedure by trained and registered staff after consulting the NVS.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidance on wild bird surveillance procedures, including organisational specific guidelines, collaborators in mainland Europe who undertake a similar surveillance protocol and recent literature on seroprevalence and viral screening in wild passerine birds.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The project licence holder will check the 3Rs website (https://www.nc3rs.org.uk/) and other accessible sources of information on animal surveillance and sampling.
The project license holder will ensure that all researchers are kept up to date with current guidelines and practices by having direct conversations before, during and after fieldwork, and recording evidence of these conversations through bi-annual welfare meetings. The

Named Information Officer will provide up to date guidance on active surveillance throughout the project.